UNIVERSITÉ DE SHERBROOKE

Faculty of Engineering

Department of Mechanical Engineering

THE EFFECT OF TISSUE MECHANICAL CHARACTERIZATION AND STIMULATION PARAMETERS ON LIVE TISSUE MECHANOBIOLOGICAL PROGRESSION WITH REGARD TO VISCOELASTICITY AND VISCOPLASTICITY

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Titre en français:

L'EFFET DE LA CARACTÉRISATION MÉCANIQUE ET DES PARAMÈTRES DE STIMULATION DES TISSUS SUR LEUR EVOLUTION MÉCANOBIOLOGIQUE EN REGARD AVEC LA VISCOÉLASTICITÉ ET LA VISCOPLASTICITÉ

TO MY MOTHER

Résumé

La caractérisation des tissus est une étape majeure dans les études mécanobiologiques. En effet, à l'aide des méthodes de caractérisation, la qualité des tissus, soit la combinaison des propriétés structurelles, compositionnelles et mécaniques, peut être déterminée. Ce projet de maîtrise focalise sur les méthodes de caractérisation mécanique pour les études *in vitro* en bioréacteur. À travers toutes les méthodes de caractérisation mécanique, nous proposons l'utilisation de celles qui sont : 1) non-destructives (i.e. qui offrent la possibilité de réaliser d'autres essais de caractérisation après les essais de caractérisation mécaniques) et 2) en-ligne (i.e. qui permettent l'observation de la progression des tissus durant l'expérimentation, et ce, sans devoir déplacer les spécimens d'une machine vers une autre). Toutefois, la caractérisation mécanique non-destructive en-ligne soulève la question à savoir si cette méthode d'observation utilisée durant l'expérimentation modifie l'évolution des tissus dans le temps.

Ainsi, le but de ce projet de maîtrise était d'approfondir nos connaissances sur les paramètres qui pourraient affecter la qualité des tissus conjonctifs mous durant une expérimentation *in vitro* en bioréacteur. Ceci passe par une meilleure compréhension de la viscoélasticité et viscoplasticité, deux comportements clés des tissus, qui affectent l'impact de ces paramètres sur la réponse des tissus vivants à des stimuli biophysiques. Donc, les deux objectifs de ce projet étaient :

1. De revoir la littérature portant sur deux comportements mécaniques des tissus, soient la viscoélasticité et la viscoplasticité, et la façon avec laquelle ils affectent l'évolution des tissus sous stimuli biophysiques;

2. D'investiguer si l'utilisation d'essais diagnostiques d'amplitude physiologique pour quantifier les propriétés mécaniques des tissus peut affecter leur évolution dans le temps.

Dans ce mémoire, nous expliquons que la viscoélasticité et la viscoplasticité des tissus proviennent de la structure et de la composition de la matrice extracellulaire. Nous décrivons également la façon avec laquelle ces comportements affectent la compétition dynamique entre la réparation, la dégradation enzymatique et la dégradation mécanique de la matrice extracellulaire sous stimuli biophysiques. De plus, nous spécifions des paramètres de stimulation, tels que le type de contrôle ou l'histoire des stimuli, qui pourraient affecter l'évolution des tissus en réponse à des stimuli biophysiques à cause de la viscoplasticité et viscoélasticité.

Aussi, nous relatons les résultats d'une expérimentation de trois jours réalisées sur des tendons fraîchement extraits pour investiguer si l'application d'essais de relaxation de contrainte d'amplitude physiologique affecte l'évolution des tissus sous stimuli mécaniques. Nous avons regroupé les tendons selon le protocole de caractérisation (0 ou 24 essais de relaxation d'amplitude physiologique chaque jour) et nous avons comparé l'évolution des groupes dans le temps. Les essais de relaxation de contraintes d'amplitude physiologique ont modifié l'évolution des tendons en réponse aux stimuli

mécaniques *in vitro*. De façon générale, le module pointe a augmenté dans le temps pour le groupe de 0 essai de relaxation de contrainte alors qu'il a d'abord diminué puis légèrement augmenté pour le groupe de 24 essais de relaxation de contrainte chaque jour. La différence entre les deux groupes était significative. Donc, l'insertion d'essais de relaxation de contrainte d'amplitude physiologique pendant les périodes de repos entre les stimuli mécaniques peut influencer l'évolution des tissus dans le temps.

Nous concluons qu'il importe de tenir compte de la viscoélasticité et de la viscoplasticité des tissus lors du développement d'un protocole de stimulation pour une étude en bioréacteur ou encore pour une application clinique.

Mots clés : tissus conjonctifs mous, mécanobiologie, évolution des tissus, propriétés des tissus, protocole de caractérisation, viscoélasticité, viscoplasticité, en-ligne, non-destructif

Abstract

Tissue characterization is a major step in tissue mechanobiological studies. By characterization methods, tissue quality i.e. the combination of tissue structural, compositional and mechanical properties, is determined. This research focuses on mechanical characterization methods. Among all mechanical characterization methods, we propose those ones which are: 1) Non-destructive, (i.e. that reserves the capability of doing other characterization tests at the end of mechanical test; and, 2) In-line, (that enables tissue progression observation during experiment, and without transferring the specimen from one apparatus to another). However, in-line characterization raises the question of whether conducting tissue observation methods during experimentation modifies tissue progression over time.

Therefore, the purpose of this study was to deepen our knowledge about the parameters which could affect tissue quality during mechanical testing. This requires a better understanding of viscoelasticity and viscoplasticity, two key behaviors of tissue, affecting the impact of these parameters (e.g. tissue quality, stimulation parameters) on the response of live tissue to biophysical stimuli. Thus, the objectives of this study were:

1. To review the literature to find information about two mechanical behaviors of tissue i.e. viscoelasticity and viscoplasticity, and the way they affect tissue properties

2. To investigate whether diagnostic tests, as mechanical characterization tests to observe tissue properties, affect tissue progression

We explain that viscoelasticity and viscoplasticity of tissue originate from structure and components of the extracellular matrix. We also describe the way they affect tissue dynamic competition between repair, enzymatic degradation and mechanical degradation of the extracellular matrix. Moreover, we specify some tissue stimulation parameters, such as stimulation control type or stimulus history, which could affect tissue progression in response to biophysical stimuli because of viscoelasticity and viscoplasticity.

Moreover, by conducting a series of 3-day experiments on freshly extracted tendons, we investigated whether applying "stress relaxation" tests at physiological amplitudes affects tissue response. We divided the tendons into two groups based on the characterization protocol (24 and 0 stress relaxation tests each day), and compared the progression of these groups over time. The stress relaxation tests at physiological amplitude modified tissue response to mechanical stimuli *in vitro*. In general, the modulus increased for 0 stress relaxation tests, while it first decreased and then increased slightly for 24 stress relaxation tests each day. The difference of mechanical properties between the two groups was significant. Therefore, applying stress relaxation tests at physiological amplitude during the rest periods between mechanical stimuli can affect live tissue progression over time.

Therefore, it is essential to take into account the viscoelasticity and viscoplasticity of tissue while developing a stimulation protocol for bioreactor studies or clinical applications.

Keywords: mechanobiology, tissue progression, tissue properties, characterization protocol, mechanical characterization, viscoelasticity and viscoplasticity, in-line, non-destructive.

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1. Introduction

Mechanobiology is the science studying tissue remodeling in response to physical/mechanical environmental stimulation (van der Meulen and Huiskes 2002). The major contributors to mechanobiology are: mechanical loading, the mechanisms by which cells could sense mechanical loading (mechanotransduction), cell response to received biophysical signals, and tissue progression based on mechanical loading and cell response.

Mechanobiology may play a major role in preventing and healing mechanically based tissue disorders. In addition, improvement of the function of engineered tissues depends on progress in mechanobiology (van der Meulen and Huiskes 2002).

A major step in mechanobiological studies is tissue characterization. Tissue characterization includes the methods which extract information about tissue quality i.e. compositional, structural, and mechanical properties of tissue. As it is observed in Chapter 2 (literature review), different characterization methods exist and are used in different laboratories. Unfortunately, most laboratories use destructive methods for mechanical characterization at the end of the experimental protocol. Therefore, by the end of experiment, no complementary characterization of compositional and structural properties can be conducted on tissue.

In our view, among all available methods for tissue mechanical characterization, in-line non-destructive tests have more advantages. With in-line monitoring, the data during experimentation are available at regular intervals thus tissue progression over time can be monitored. Moreover, since the stimulation and characterization methods are conducted inside the same apparatus (for in vitro studies), the errors and damages which may occur with transferring the samples from one apparatus to another are eliminated. In addition, in non-destructive tests conducted at regular intervals, samples can be self-compared, thus reducing the number of samples and animals are needed. The data acquired from these self-compared samples are thus more reliable because

there is no intra-sample variability. Finally, at the end of non-destructive tests, other complementary characterization tests can be conducted.

All the bioreactor experimentations at Biometiss[1] have been carried out based on in-line non-destructive characterization protocols. For most of them, tissue stimulation protocols (a series of operations applied on tissue during experiment including: preloading, preconditioning, cyclic loading-unloading, resting, etc.) have been designed based on the same standards. For example preconditioning, amplitude and duration of preloading and stress-relaxation tests and mechanical stimuli, duration of resting between mechanical stimuli, etc are standardized.

Although it is very useful to have the information of tissue progression over time, it raises a concern. Does tissue react to our characterization method and does it alter its progression over time? In other words, does the method used to observe tissue during the experiment affects experimental results?

These concerns were questioned in the cell mechanics field by (Bao and Suresh 2003). The authors asked this paradox: "how can we measure the mechanical behaviour of living cells if they react to our measurement tools? To our knowledge, this is the first time this topic was discussed at the tissue level. This issue is very important because the effect, of methods used to characterize tissue, on tissue response, could make the experimental result un-reliable.

The objectives of this research project were:

1. **To review the literature about two key behaviors of fibrous load bearing tissues (i.e. viscoelasticity and viscoplasticity) and explain how they affect live tissue response to mechanical characterization;**

The effect of viscoelasticity and viscoplasticity on tissue response is a very important subject which must be taken into account for treating and preventing tissue disorders and improving tissue quality based on mechanobiology. For example, since fibrous load

[1] The laboratory at University of Sherbrooke working in the field of Mechanobiology.

bearing tissues are viscoelastic and viscoplastic, the response of these tissues with two different qualities (e.g. healthy vs. damaged) to an identical mechanical stimulation could be different (e.g. constructive vs. destructive). Moreover, because of viscoelasticity and viscoplasticity of tissues, changes in stimulation parameters, (e.g. changes in nature of loading: stress vs. strain or static vs. cyclic) could make an essential difference in tissue responses.

2. **To investigate if diagnostic tests conducted at regular intervals affect live tissue response or not.**

Either "stimulation protocol" or "diagnostic test", i.e. mechanical tests interspersed at time intervals during the stimulation protocol used to observe tissue progression over time, could be used as tissue mechanical characterization test. In either of these methods, some mechanical variables are measured (e.g. load and/or displacement) or calculated (e.g. stiffness and/or hysteresis). These variables represent the tissue mechanical quality. If we measure or calculate these variables at regular intervals, we will have tissue progression over time.

Using diagnostic tests to evaluate tissue progression over time has an advantage over using stimulation protocols in which parameters such as frequency or amplitude could change between different experiments, in different laboratories, in different days, and on different tissues. Using diagnostic tests (e.g. stress relaxation tests) makes it possible to define the "diagnostic test", in which parameters such as frequency or amplitude remain constant between different experiments, as a "reference standard" in all experiments. However, there is a concern whether diagnostic tests affect tissue response or not.

With these objectives in view, the thesis contains two articles, one for each objective, and is divided into six Chapters. In Chapter 2, compositional, structural, and mechanical tendon properties are briefly explained. It is worth noting that the hypotheses and the discussions are not limited to tendons but are attributed to all fibrous-load- bearing tissues. Some methods used in literature for compositional, structural, and mechanical

characterization are then presented to have an overview of the characterization methods used in tissue quality.

In Chapter 3, the origin of viscoelasticity and viscoplasticity in tissues and the way they affect live tissue properties are explained. This chapter has been submitted as a review article.

Another article has been written to fulfill objective 2 and is presented in Chapter 4. As reported in this manuscript, live healthy tendons were subjected to physical stimuli at physiological amplitude *in vitro*. Stress-relaxation tests were conducted at regular intervals to observe tissue progression over time. We investigated if stress-relaxation tests affect tissue progression or not.

In Chapter 5, unpublished results are presented. These results include methods we used at Biometiss to characterize tendon structural ECM and cellular quality using microscopic images.

Finally, a discussion is presented in Chapter 6 (in both English and French), drawing conclusions about this work and proposing future studies.

2. State-Of-The-Art

This chapter reviews important literature relative to the presented master's project. It is divided in two sections.

In the first section, we will introduce compositional, structural and mechanical properties of tendons. A combination of these properties could be defined as tissue quality. In the study of tendon physiology, pathology, or healing an important step is determining tissue quality. One of the most important fields of tissue study which needs tissue quality information is mechanobiology. We will therefore end the first section with a brief explanation of mechanobiology but also of mechanotransduction, the important mechanisms which are involved in mechanobiological remodeling of tissue.

In the second section, we will review some literature to highlight the methods of gathering information regarding tissue quality, i.e. characterization methods. The mentioned characterization methods are the ones which have been mostly used in the literature.

2.1 Tendon compositional properties

Tendons are those connective tissues which connect muscle to bone. Tendons generally consist of the ECM and cells (tenocytes) which are, respectively, inert and active components of tendons. Although these two components are in a closed and bidirectional interaction together, we can devote the mechanical behavior of the tendon mostly to the ECM, and consider cells as responsible for remodeling of tissue (or mainly the ECM) (Kalson, Holmes et al.).

The ECM contains almost 70% water and 30% solid (Margareta Nordin and L. 2001). Solid part contains mostlycollagen fiber, some elastin, as well as ground substance (Margareta Nordin and L. 2001).

Collagen and elastin are structural proteins of the ECM. In fact, the biomolecules in the ECM could be divided into three subgroups: 1) structural proteins like collagen and elastin, 2) specialized proteins like fibronectin, and 3) proteoglycans (Xu 2008).

Collagen is the most important component and provides the strength of tendons against applied tensile loads. There are 19 different kinds of collagens of which the most abundant type in tendons are type I collagens. Their parallel alignment along the tendons let them resist tensile load in this direction.

Elastin fibers , the smallest representatives of the ECM, represent 1-2% of dry weight of tendon. These proteins are associated with collagen fibers not only to withstand tensile loads, but to provide elasticity to tendons (Margareta Nordin and L. 2001; Sharma P 2006).

Ground substance constitutes the remainder. It consists mainly of proteoglycans, matrix glycoproteins and water (Chun k 2003). Glycosaminoglycans, a major component of proteoglycans, are large negatively charged and hydrophilic molecules. Because of the repulsive force between two negative charges, glycosaminoglycans offer tissue resistance to compression (Chun k 2003) and may play a role in the spacing of collagen fibres (Hansen, Weiss et al. 2002). They also capture the majority of the extracellular water (Margareta Nordin and L. 2001) and create a gel-like substance in the collagenous matrix (Margareta Nordin and L. 2001). Finally, it is believed that molecules from the ground substance play an important role in relative motions of collagen fibrils in mechanically loaded tendons [(Mosler, Folkhard et al. 1985); (H R C Screen 2004)].

2.2 Tendon structural properties

The hierarchical structure of a healthy tendon is shown in Figure 2-1. Tropocollagens (collagen molecules) unite into collagen fibrils, collagen fibers[2], subfascicles (primary bundles), and fascicles (secondary bundles). Several fascicles constitute tertiary bundles (Liu, Ramanath et al. 2008).

[2] There have been some misunderstandings in literature regarding using "fiber" and "fibril". In some texts, these two terms have been used interchangeably.

Primary, secondary and tertiary fiber bundles are covered by a thin layer called endotenon and the whole tendon is surrounded by another thin layer called epitenon (Sharma P 2006).

Tendon cells (tenocytes), which are responsible for production of collagen fibers and of ground substance, are located between fibers. They have an elongated shape when observed in the tendon's longitudinal orientation (Margareta Nordin and L. 2001). Whereas in cross-section, they appear as star-shaped cells (C M McNeilly 1996).

Some structural criteria to classify the quality include cell shape, collagen organization, cell-ECM interaction, cell density, etc. Methods could be divided into three groups: qualitative, semi-quantitative, and quantitative which are introduced in the three following sections.

We explain these methods, since they are used in clinical applications. Moreover, in our *in vitro* experimentations, we use these methods to compare the structural quality of different groups of samples.

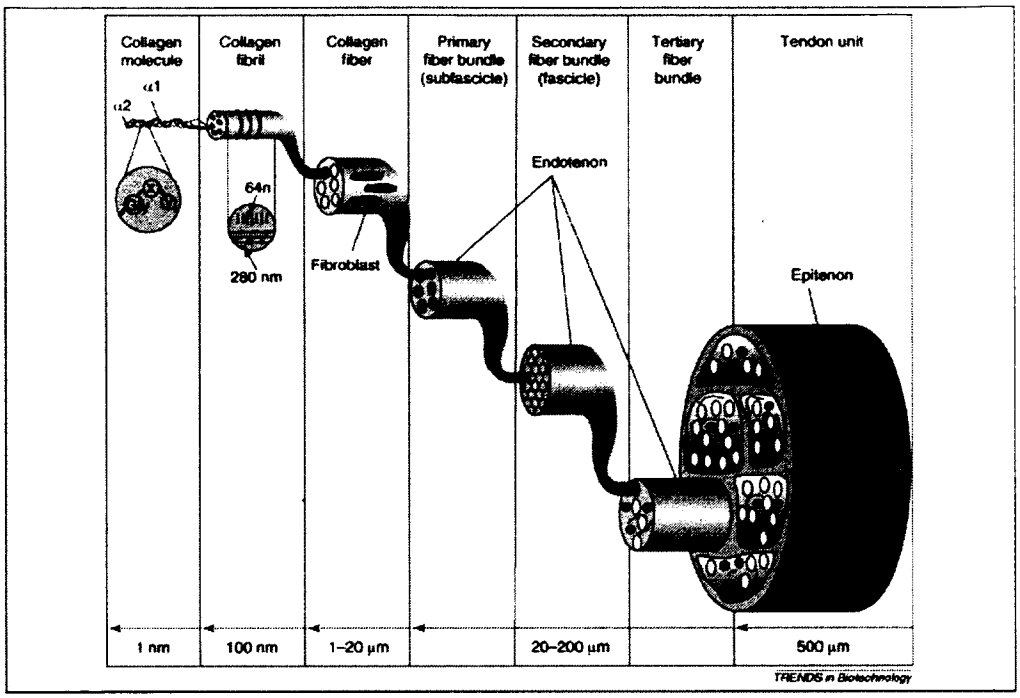

Figure 2-1: Schematic structure of a normal tendon (Liu, Ramanath et al. 2008)

2.2.1 Qualitative characterization

One method for the characterizing tissue quality is histology, i.e. characterization of tissue structure using microscopic images. Microscopic images are mainly from light (optical) microscopy (OM), and electron microscopy (EM). They both have an objective lens to magnify the structures and are used in biology and material science fields (Alberts B 1994). In OM, a photon beam is radiated to the objective lens to visualize the purpose structure, while in EM, the radiated beam is made up of electrons (Keith Wilson 2005). This difference in the type of radiated beams makes each microscope appropriate for special purposes. The electron microscope provides a much higher resolution and magnification than optical microscope. Therefore, to resolve very small objects, e.g. small molecules with approximate size of 1 nm, EM should be used.

Table 2-1 demonstrates important structural characteristics of healthy and tendinopathic tendons (Xu 2008).

Table 2-1: Comparison of normal and tendinopathic tendon by microscopy (Xu 2008)

Findings	Macroscopic	Optical microscopy (longitudinal sections)	Electron microscopy (transversal sections)
Normal tendon	Brilliant white Fibroelastic Firm texture alignment	Organized parallel collagen bundles Spindle shape tenocyte nuclei Nuclei parallel alignment	Densely packed collagen fibers Uniform in diameter and orientation of collagen fibers
Tendinopathic tendon	Grey or brown Tissue is thin, fragile and disorganized Loose texture	Disorganized collagen bundle Increased ground substance consisting of proteoglycan and glycosaminoglycan (GAG) Large mucoid patches and vacuoles between fibers[3] (Figure 2-2) Round with darker-staining tenocyte nuclei Markedly increased number of tenocyte nuclei with loss of parallel alignment Increase of vascular and nerve ingrowths	Angulation (Figure 2-3), bubble formation (Figure 2-4) of collagen fibers Variation in the diameters and orientation of collagen fibers Hypoxic [4](Figure 2-5) changes in tenocyte (lipid vacuoles[5], enlarge lysosomes [6]and degranulated endoplasmic retinaculum[7] (Figure 2-5))

[3] One type of tendon degeneration. Accumulation of large mucoid patches and vacuoles filled with GAGs and proteoglycans between collagen fibers Peter A. Huijbregts, M., MHSc, PT Scott E. Smith, MSc, OT (1999). "Tendon Injury: A Review." The Journal of Manual & Manipulative Therapy 7: 71-80.

[4] One type of tendon degeneration which is deprivation of adequate oxygen.

[5] Lipid accumulation

[6] Lysosomes are one of subcellular components which contain waste-breaking enzymes.

[7] The endoplasmic reticulum (ER) is a continuous membrane which has many different functions such as : translocation of proteins across the ER membrane; the integration of proteins into the membrane; etc. Gia K. Voeltz, M. M. R. (2002). "Structural organization of the endoplasmic reticulum." EMBO reports 3(10): 944-950.

Figure 2-2: Light microscopy of a ruptured Achilles tendon from a twenty-nine-year-old woman. The arrow shows the thin and fragile collagen fibers, and the star shows the large vacuoles among the fibers. (Kannus P 1991)

Figure 2-3: Transmission electron microscopy of a ruptured extensor pollicis longus tendon from a sixty-four-year-old woman. The arrow shows the angulation of the collagen fibrils (Kannus P 1991)

Figure 2-4: Transmission electron microscopy of a ruptured Achilles tendon from a thirty-four-year-old man. The arrow shows bubble formation involving some fibrils. (Kannus P 1991)

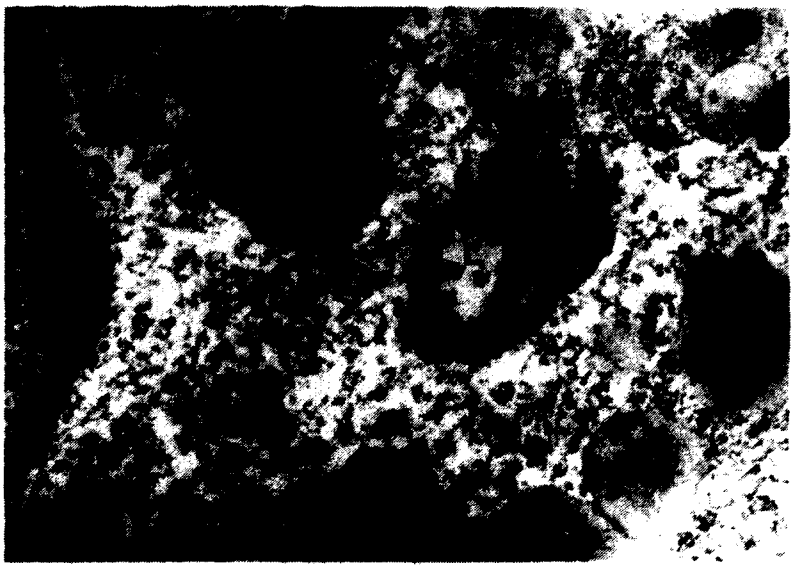

Figure 2-5: Transmission electron microscopy of a ruptured Achilles tendon from a thirty-three-year-old man. The image shows a high-level hypoxic degenerated tenocyte which includes lipid vacuoles (LV), enlarged lysosomes (L), and degranulated endoplasmic retinaculum (E) (Kannus P 1991)

2.2.2 Semi-quantitative characterization

Most tissue histological characterization studies do not quantify the properties, but use description of the changes from healthy to damaged tissue histology (Nicola Maffulli 2008). The method of description of structural changes can lead to inadequacy in classifying the different levels of tissue injury. This misunderstanding and uncertainty about tissue condition may result in inconsistent diagnosis between specialists (Nicola Maffulli 2008).

To avoid this uncertainty in diagnoses by different specialists, some scoring methods have been suggested to be used to classify the tendinopathic tendons (Nicola Maffulli 2008). These methods were developed for clinical applications so they score the level of tendinopathy. There are two kinds of such scoring systems: Movin and Bonar systems. In each method they score specific variables which evaluate various aspects of tissue quality. Both of these methods were created for classifying OM images of longitudinal tendon section.

The variables included in the Movin scaling method are: (1) fiber structure, (2) fiber arrangement, (3) rounding of the nuclei, (4) regional variations in cellularity, (5) increased vascularity, (6) decreased collagen stainability, and (7) hyalinization. For each variable, the score could be 0 (normal tendon) to 3 (the most abnormal appearance detectable) (Longo, Franceschi et al. 2008). Therefore the total score of each sample could vary between 0 (normal tendon) to 21 (the most severe abnormality detectable).

For example (Longo, Franceschi et al. 2008) used Movin scoring method to investigate the histological changes of Supraspinatus tendon in rotator cuff tears. They classified light micrographs of normal and injured tendons based on Movin scoring scales (Figure 2-6 to Figure 2-9).

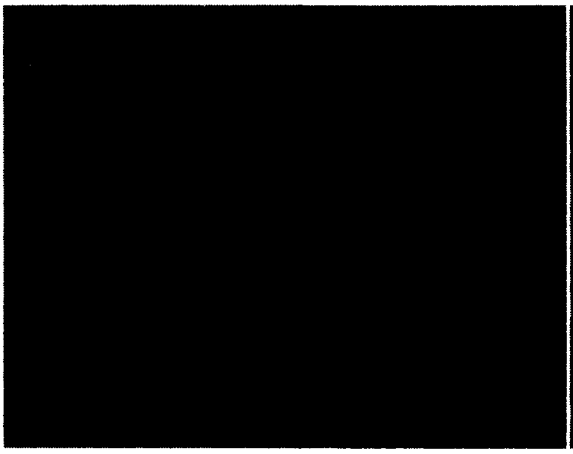

Figure 2-6: Hematoxylin and eosin stain of a control supraspinatus tendon in a 71-year-old man. Fiber structure, 0; fiber arrangement, 0; rounding of the nuclei, 0; regional variations in cellularity, 1; increased vascularity, 0; decreased collagen stainability, 0; hyalinization, 0. Total score: 1

Figure 2-7: Hematoxylin and eosin stain of supraspinatus tendon harvested from the intact middle portion of the tendon between the lateral edge of the tendon tear and the muscle-tendon junction in a 62-year-old woman. Fiber structure, 2; fiber arrangement, 2; rounding of the nuclei, 3; regional variations in cellularity, 2; increased vascularity, 0; decreased collagen stainability, 1; hyalinization, 0. Total score: 10

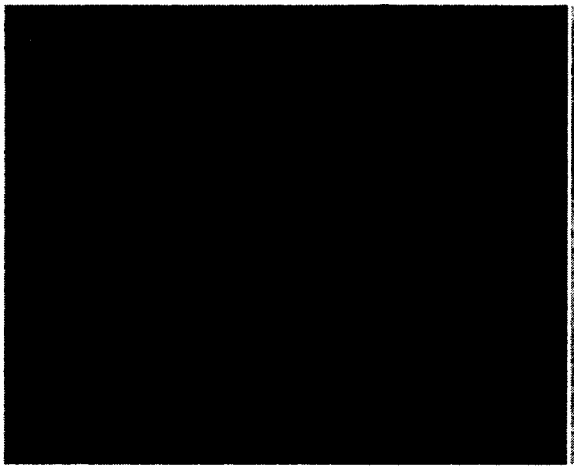

Figure 2-8: Hematoxylin and eosin stain of supraspinatus tendon harvested from the intact middle portion of the tendon between the lateral edge of the tendon tear and the muscletendon junction in a 53-year-old man. Fiber structure, 2; fiber arrangement, 2; rounding of the nuclei, 1; regional variations in cellularity, 1; increased vascularity, 1; decreased collagen stainability, 2; hyalinization, 0. Total score: 9

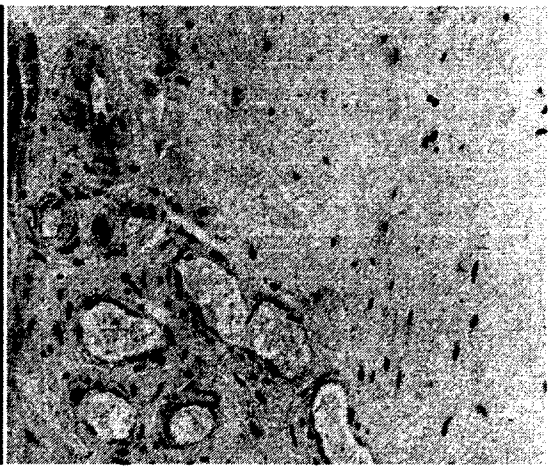

Figure 2-9: Hematoxylin and eosin stain of supraspinatus tendon harvested from the intact middle portion of the tendon between the lateral edge of the tendon tear and the muscletendon junction in a 59-year-old man. Fiber structure, 2; fiber arrangement, 2; rounding of the nuclei, 1; regional variations in cellularity, 2; increased vascularity, 3; decreased collagen stainability, 2; hyalinization, 0.Total score: 12

The variables included in the Bonar scaling method are: (1) tenocytes; (2) ground

Table 2-2: Semi-quantitative scoring (Bonar scale) (Cook, Feller et al. 2004)

Grade	0	1	2	3
Tenocytes	Inconspicuous elongated spindle shaped nuclei with no obvious cytoplasm at light microscopy	Increased roundness: nucleus becomes more ovoid to round in shape without conspicuous cytoplasm	Increased roundness and Size; the nucleus is round. slightly enlarged and a small amount of cytoplasm is visible	Nucleus is round, large with abundant cytoplasm and lacuna formation (chondroid change)
Ground substance (alcian blue and colloidaliron stains)	No stainable ground substance	Stainable mucin between fibers but bundles still discrete	Stainable mucin between fibers with loss of clear demarcation of bundles	Abundant mucin throughout with inconspicuous collagen staining
Collagen (with and without polarized light)	Collagen arranged in tightly cohesive well demarcated bundles with a smooth dense bright homogeneous polarization pattern with normal crimping	Diminished fiber polarization; separation of individual fibers with maintenance of demarcated bundles	Bundle changes; separation of fibers with loss of demarcation of bundles giving rise to expansion of the tissue overall and clear loss of normal polarization Pattern	Marked separation of fibers with complete loss of architecture
Vascularity	Inconspicuous blood vessels coursing between bundles	Occasional cluster of capillaries, less than 1 per 10 high power fields	1-2 clusters of capillaries per 10 high power fields	Greater than 2 clusters per 10 high power fields

substance; (3) collagen; and (4) vascularity. For each variable the score could be 0 (normal tendon) to 3 (the most abnormal tendon detectable)(Nicola Maffulli 2008). Therefore, the total score of each sample could vary between 0 (normal tendon) and 12 (the most severe abnormality detectable) (Table 2-2).

Using either the Movin or Bonar method leads to similar results (Nicola Maffulli 2008). By using either of these methods, one is capable of quantifying the appearance of normal and tendinopathic tendon.

2.2.3 Quantitative characterization

There are some methods to quantify tissue structural properties. Using image processing techniques, one could obtain various measurements in images. For example, (Parent G, Langelier et al. 2011) measured space between fibers using Vision assistant software, (Version 7.1 National Instrument, Austin, TX, USA). They chose three regions of interest (ROIs) for each of their microscopic images (OM) and found the spaces between the fibers by contrast dividing the objects into 2 categories: fibril (black), and space (red). Space density was calculated by dividing the number of red pixels by the number of pixels in the image. They also evaluated the mean area of the spaces, i.e. average of the number of connected red pixels using the same software

They have also calculated fibril density through transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images.by chosing three ROIs in each image, and finding the fibril pixels from background pixels using bottom-hat filtering in Matlab (Version 7.5, Mathworks, Natick, USA). Fibril density was calculated by dividing the number of fibril pixels by the total number of image pixels.

2.3 Tendon mechanical properties

The stress-strain diagram in Figure 2-10 shows the mechanical behavior of rat tail tendons from a study of (Arnoczky, Lavagnino et al. 2007). Although values are specified for rat tail tendons, the general trends are the same for all kinds of tendons. As can be seen in this figure, the "physiologic range" includes a toe region and part of linear region. In this range, collagen fibrils begin to un-crimp and then they are

stretched by increasing load. Near the end of the linear region, isolated collagen fibrils begin to fail (Arnoczky, Lavagnino et al. 2007), and stress-strain curve enters to the region specified as "overuse injury" region which is the region of isolated collagen fibril microdamages. In this region, straightening of collagens is continued and interfibrillar sliding and shear between collagen fibrils produces a non-linear load-deformation behavior of the tendon (Arnoczky, Lavagnino et al. 2007). Some collagen fibrils are damaged before others until a complete "tendon rupture" occurs in the last region of stress-strain curve (Arnoczky, Lavagnino et al. 2007).

Therefore, collagen fibers properties, their crimp structure and their failure level, play a significant role in biomechanical behavior of tendon which are to support and transmit tensional load.

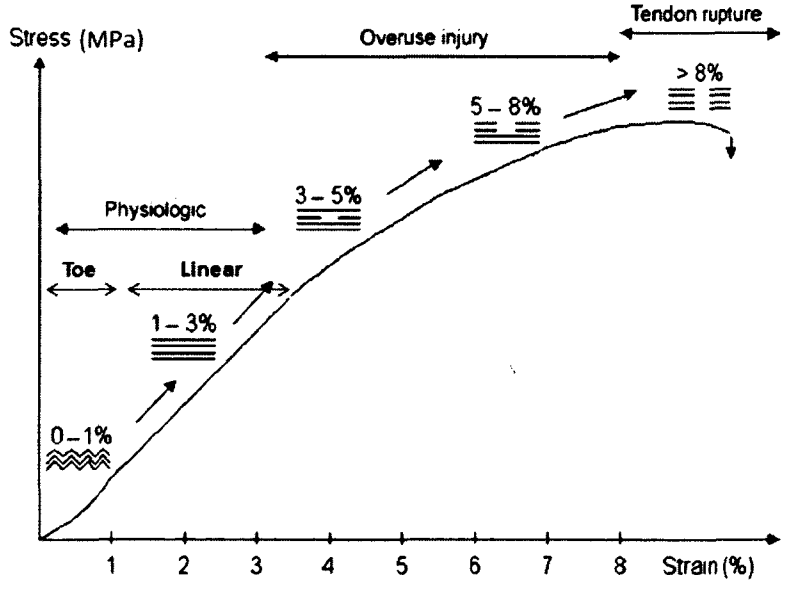

Figure 2-10: Stress-strain curve demonstrating the mechanical properties of normal tendon (Arnoczky, Lavagnino et al. 2007)

A tendon is not a pure elastic material and displays viscoelastic behaviors. It has the properties of both elastic and viscous material, which means the rate of loading has an effect on tendon behavior. Also, energy is lost during strain-loading, and thus the loading and unloading curves will not be identical, a phenomenon called "hysteresis".

Two other phenomena originating from viscoelasticity are stress relaxation and creep (Margareta Nordin and L. 2001). Stress relaxation is reducing (relaxing) stress under constant strain, whereas creep is increasing strain during a constant load. Related information will be presented in Chapter 3.

Under certain conditions of loading and tissue quality, tendons also show viscoplastic behavior. The tendon is not able to get back to its initial length after unloading since it undergoes some plastic/permanent deformations. We will explain this in further detail in Chapter 3.

Up to now, compositional, structural, and mechanical properties of tendon have been introduced since a tendon is a live system which progresses with time, these properties can undergo some changes. Some factors which can affect tendon properties are aging, diseases, and changes in environmental loading. As mentioned earlier, cells represent the active component of tendons, and therefore tendon progression depends on cell response to these factors, among which only environmental loading will be discussed in this thesis. It is important to know how cells sense the environmental loading and how they respond to it. In the following section, this subject will be briefly described.

2.4 Mechanobiology and mechanotransduction

Since many tissue disorders result from mechanical overloading, it is very important to study the relationship between mechanobiological stimulation and tissue progression. Mechanobiology discusses these issues. In other words, mechanobiology is the science which studies the remodeling of the tissues in response to physical loading (van der Meulen and Huiskes 2002). Tissues are constructed and remodeled by cells. Therefore, mechanotransduction is the mechanisms by which loading could be sensed by the cells.

Examples of mechanotransduction mechanisms are: cell deformation, nucleus deformation, cytoskeleton, stretch activated channels, and primary cilium (Wang 2006). Through these mechanisms, mechanical stimulation is converted into biochemical signals. Mechanical stimulation, applied to the ECM can damage it. It can further undergo more damages or can be repaired by cellular activity Biochemical signals,

resulted from converting mechanical stimulation through mechanotransduction mechanisms, are detected by cells. Cells can respond differently depending on stimulation. they can "repair" tissue by producing and maintaining the collagens (Devkota, Tsuzaki et al. 2007; Kjær, Langberg et al. 2009) or cause "degradation" by secreting of collagen degradable enzymes i.e. proteases (Arnoczky, Lavagnino et al. 2007; Devkota, Tsuzaki et al. 2007; Cousineau-Pelletier and Langelier 2009). Therefore, mechanobiology plays a major role in establishing tissue homeostasis.

Mechanobiology and mechanotransduction will be discussed in more depth in Chapter 3.

2.5 Literature review of characterization methods

Although mechanical properties play an important role in tendon functionality (Duenwald-Kuehl, Lakes et al. 2012), compositional, and structural properties are also of great value in providing complementary information on tissue quality. In fact, compositional, structural, and mechanical properties are in a close relation, therefore, studying tendon biomechanical and mechanobiological behavior, not only is important for characterizing mechanical properties, , but it is also important for characterizing compositional and structural properties.

Tissue quality can take different values depending on the properties of the tissue. For example, tissue could be healthy vs. damaged. It should be noted that tissue quality affects the cellular response of the tissue. This will be explained in Chapter 3.

Investigating tissue quality is important to evaluate tissue progression over time. For example, to evaluate the efficiency of a training protocol we need to compare the tissue quality before and after the training. Investigating tissue quality is therefore unavoidable for further studies of tendon mechanobiology.

In the following section, literature introducing methods which have been used to characterize tissue properties will be reviewed.. Presented articles are divided into three categories corresponding to the type of experiment conducted: 1) *in vitro* with live cells, 2) *in vitro* with dead cells, and 3) *in vivo*. For each article, a table which

summarizes the tissue quality characterization techniques for tissue quality is presented. The tissue characterization table is divided into three categories: mechanical characterization, structural characterization, and compositional characterization. Each category, in turn, is divided into two subcategories. In the first subcategory, the conducted test will be explained (e.g. in-line, destructive). In the second subcategory, the information related to data acquired from the conducted test will be presented. The hypothesis and results of the article are also explained briefly. At the end of this section, a discussion about these techniques is provided.

Some of the expressions used to describe mechanical tests which might be less familiar to the reader, are defined here:

- *In-line characterization:* the mechanobiological experimentation (to examine the impact of a loading regime on tissue progression), and characterization testing (to determine tissue quality) are conducted in the same apparatus. Therefore, the information about tissue mechanical properties are available at regular time intervals during the experiment without changing the tissue from one apparatus to another.

- *Non-destructive characterization:* the characterization does not lead to tissue damage or failure. Therefore, other characterization tests could be conducted after non-destructive characterization.

2.6 *In vitro* experimentation on tendons with live cells

Article 1. Distributing a fixed amount of cyclic loading to tendon explants over longer periods induces greater cellular and mechanical responses (Devkota, Tsuzaki et al. 2007)

Hypothesis/Objective:

I. Magnitude: High-magnitude cyclic loading would cause injury, but not low-magnitude cyclic loading.

II. Duration: For a fixed number of cyclic loading on tendon, the longer the period of loading, the greater the mechanical and cellular responses of tendon.

Table 2-3: Summary of characterization tests conducted in this article

Mechanical characterization		**Structural characterization**	**Compositional characterization**	
Test	**Measured variable**	None	**Test**	**Analysis**
In-line Non-destructive Dynamic loading Stress-controlled	Dynamic strain[8] Stiffness		Destructive	Hydroxyproline content assay (determining collagen content) Sulfated GAGs content assay (determining proteoglycan content)
			Non-destructive	Immunoassay kit on media (determining inflammatory mediators, PGE2) Azocolle procedure on media (collagenase content)
In-line	Static strain		None	

[8] Dynamic strain = $\frac{\text{Peak} - \text{trough}}{\text{trough}}$ displacement, throughout loading

Non-destructive Creep Stress-controlled				
Off-line[9] Destructive Failure Strain-controlled	Strain at failure Energy density[10]		None	

Results

The loading was conducted on two groups and in four regimens. Figure 2-12 shows the schematic of the loading regimens.

- Dynamic and static strain accumulations were larger in "High-magnitude/Long-loading" compared to "High-magnitude/Short-loading" groups.
- Static strain accumulation was greater in "Low-magnitude/Long-loading" compared to "Low-magnitude/Short-loading" groups. These results show the effect of loading time on the tissue response.

[9] Failure test was performed on dead tissues, since the tissues were first frozen (at -9°C) and then thawed.
[10] The amount of energy tendons absorb before failing.

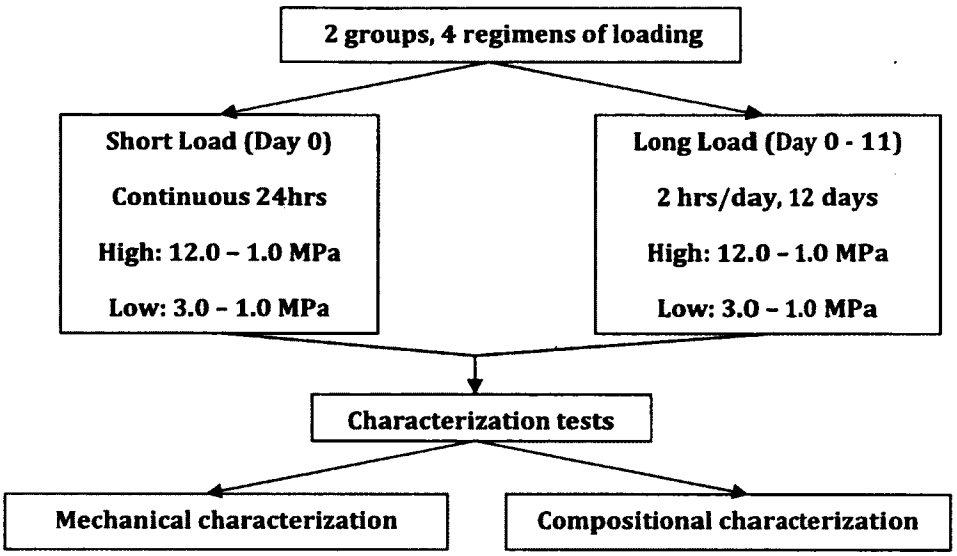

Figure 2-12: Schematic of the loading and assaying of the tendon

- However for dynamic strain, there was no difference between "Low-magnitude/Long-loading" and "Low-magnitude/Short-loading" groups. Taking into account this result, along with the result from static strain, suggests that time is not the only factor affecting tissue response. As it is observed from mechanical and compositional analyses, loading magnitude also plays an important role in tissue response.

- The results from failure test did not show a definite effect of cellular response on tendon properties. The properties were either time dependent or load-magnitude dependent but not both. The "High-magnitude/Long-loading" did not consistently produce the most inferior results expected. The authors suggest the effect of the difference of cross-sectional areas of tendons as the reason of these uncertain failure results.

Article 2. Biomechanical response of collagen fascicles to restressing after stress deprivation during culture. (Yamamoto, Kogawa et al. 2007)

Hypothesis/Objective:

Restressing will improve the decreased properties of fascicles resulting from stress shielding.

Table 2-4: Summary of characterization tests conducted in this article

Mechanical characterization		**Structural characterization**[11]		**Compositional characterization**
Test	**Measured variable**	**Test**	**Analysis**	None
Off-line Destructive Failure Strain controlled	Tangent Modulus Tensile strength Strain at failure	Destructive Microscopy (OM)	Qualification Quantification (crimp angle, crimp length, wavelength)	

Result:

- The decrease of mechanical properties, represented by tangent modulus and tensile strength, was stopped and, in most cases reversed by applying stress after stress deprivation but none of them improved to their normal level.
- Structural characterization results were also consistent with mechanical characterization results. The crimp morphology of fascicles was not recovered to original levels, after restressing.

[11] Samples were separated for mechanical and structural characterization.

Article 3. Relative contribution of mechanical degradation, enzymatic degradation, and repair of the extracellular matrix on the response of tendons when subjected to under- and over- mechanical stimulations in vitro.(Cousineau-Pelletier and Langelier 2009)

Hypothesis/Objective:

Investigating the contribution of the three sub-processes of tendon response (Repair, Mechanical degradation, and Enzymatic degradation) when subjected to cyclic mechanical loading.

Tendon mechanobiological response (TMR) could be approximated as:

TMR = R – MD – ED; where

R: Repair, MD: Mechanical degradation, ED: Enzymatic degradation.

Table 2-5: Summary of characterization tests conducted in this article

Mechanical characterization		**Structural characterization**		**Compositional characterization**
Test	**Measured variable**	**Test**	**Analysis**	None
In-line Non-destructive Cyclic loading Strain controlled	Peak to peak stress	Destructive Microscopy (OM, TEM[12])	Qualification Quantification (fibril density)	

[12] Transmission electron microscopy

Result:

- In the absence of R and ED, i.e. when TMR is represented by -MD: the results from mechanical characterization, showed a fast decrease in peak stresses during experimentation time without any increase indicating tendon damage. Structural analyses supported mechanical data since they showed loosely packed and wavy collagen structure.

- In the absence of ED, i.e. when TMR is represented by R-MD, the results from mechanical characterization showed an overall increase in peak stresses during experimentation time indicating tendon improvement. Structural analyses are in well correspondence to mechanical results, since they show dense and well-oriented collagen structure.

- In the presence of all three sub-processes, i.e. when TMR is represented by R-MD-ED, the results from mechanical characterization showed a decrease of peak stresses after the initial increase. Structural analyses were consistent with these results since they showed disorganized collagen structure.

Article 4. Effect of Preconditioning and Stress Relaxation on Local Collagen Fiber Re-Alignment: Inhomogeneous Properties of Rat Supraspinatus Tendon[13]. (Miller KS 2012)

Hypothesis/Objective:

I. The greatest fiber re-alignment will occur in the toe-region at ramp-to-failure test but some fiber re-alignment will also occur during preconditioning.

II. Disorganization in collagen fiber will occur during stress-relaxation test.

III. Mechanical properties and initial collagen fiber alignment are greater at midsubstance of tendon than tendon-to-bone insertion site.

Table 2-6: Summary of characterization tests conducted in this article

Mechanical characterization		**Structural characterization**		**Compositional characterization**
Test	**Measured variable**	**Test**	**Analysis**	None
In-line Non-destructive Preconditioning Force-controlled	Grip to grip strain	Non-destructive OM with polarized light	Quantification (changes in collagen fiber re-alignment)	
In-line Non-destructive Stress relaxation Strain-controlled	Force			

[13] There is no certain reference in the article whether they worked on live or dead tissues.

In-line Both destructive and non-destructive Ramp-to-Failure Strain-controlled	Stiffness Strain at failure Stress			

Result:

- The greatest fiber re-alignment occurred during preconditioning and then at toe- and linear regions of the ramp-to-failure test.
- No collagen fiber re-alignment observed during stress-relaxation test.
- Lower moduli, more disorganizations and higher strains at insertion site than tendon midsubstance indicate that mechanics and structure of the tissue differ at different tissue locations, i.e. the tissue is not homogeneous.

2.7 *In vitro* experimentation on tendons with dead cells

In this type of experiments, i.e. tendon with dead cells, the biomechanical behavior of tendon is related to ECM since the ECM remodeling by the cells is avoided here.

Article 1. Low Stress Tendon Fatigue is a Relatively Rapid Process in the Context of Overuse Injuries. (Parent G, Langelier et al. 2011)

Hypothesis/Objective:

I. Damage progression of tendons, even with low stress, is a rapid process.

II. Compliance amplitude increases with increasing injury.

III. Damage progression affects collagen network.

Table 2-7: Summary of characterization tests conducted in this article

Mechanical characterization		**Structural characterization**		**Compositional characterization**
Test	**Measured variable**	**Test**	**Analysis**	None
In-line Non-destructive Cycling Stress controlled	Dynamic compliance Mean Strain	Destructive Microscopy (OM, TEM, SEM[14])	Qualification Quantification (Fiber density)	

[14] Scanning electron microscopy

Result:

- Strain increased with increasing levels of fatigue.
- Compliance decreased at the beginning. Thereafter, it increased with increasing fatigue levels.
- Structural characterization also showed disorganization of the collagen structure, another evidence of mechanical degradation.
- These results support the hypothesis that mechanical degradation of tendon is a very fast process even at low stresses.

Article 2. Sub rupture Tendon Fatigue Damage. (Fung, Wang et al. 2009)

Hypothesis/Objective:

Characterizing the changes in the mechanical and micro-structural properties of tendon at controlled fatigue levels.

Table 2-8: Summary of characterization tests conducted in this article

Mechanical characterization		**Structural characterization**		**Compositional characterization**
Test	**Measured variable**	**Test**	**Analysis**	None
In-line Destructive Cycling Stress controlled	Calmp-to-clamp strain Stiffness Hysteresis	Destructive Microscopy (OM, confocal microscopy)	Qualification Quantification (damage area fraction[15])	

Result:

- Strain increased significantly with increasing levels of fatigue even at lower fatigue levels. Therefore, clamp-to-clamp strain is an appropriate indicator of damage from early to late fatigue.
- Changes in stiffness and hysteresis were significant only at higher levels of fatigue.
- Structural analyses also showed disorganization in collagen structure with increasing fatigue levels.

[15] number of the pixels with tissue deformity as a percentage of number of pixels of total area

- Therefore both mechanical and microstructural characterization showed degradation in tendon properties through fatigue levels which are a result of accumulation of micro-damages.

Article 3. Corticosteroid administration alters the mechanical properties of isolated collagen fascicles in rat tail tendon. (Haraldsson, Aagaard et al. 2009)

Hypothesis/Objective:

Injecting corticosteroid reduces biomechanical properties of collagen fascicles.

Table 2-9: Summary of characterization tests conducted in this article

Mechanical characterization		**Structural characterization**	**Compositional characterization**
Test	**Measured variable**	None	None
In-line	Yield stress		
Destructive	Peak stress		
Failure	Stiffness		
Strain controlled	Strain at failure		

Result:

- The mechanical characteristics of corticosteroid-treated tendons decreased since the results showed lower yield stress, peak stress, and stiffness, in treated tendons compared to control group.
- The strain at failure remained constant between these groups.

2.8 *In vivo* experimentation

Article 1. Early response to tendon fatigue damage accumulation in a novel in vivo model. (Fung, Wang et al. 2010)

Hypothesis/Objective:

A fatigue-damaged tendon response differs from a lacerated tendon healing response.

Table 2-10: Summary of characterization tests conducted in this article

Mechanical characterization		**Structural characterization**		**Compositional characterization**	
Test	**Measured variable**	**Test**	**Analysis**	**Test**	**Analysis**
In-line Non-destructive Cycling Force controlled	Peak cyclic strain Stiffness Hysteresis	Destructive Microscopy (confocal microscopy)	Qualification	Destructive	Reverse transcription PCR (Collagen I, III, V mRNA expression)

Result:

- Mechanical characterization results demonstrated that strain increased with increasing tissue fatigue levels. This is consistent with expected changes of strain in damaged tendon from other studies and also with structural characterization results from this article (Figure 2-13).

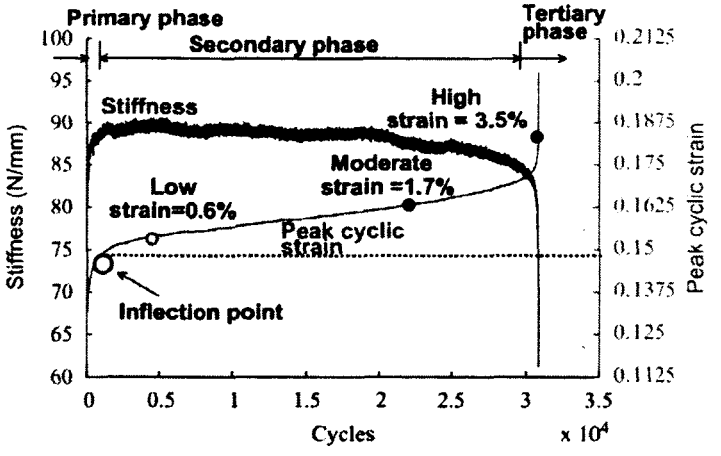

Figure 2-13: The changes of strain and stiffness during different levels of fatigue loading. As it can be viewed in the figure, strain always has an increasing pattern (at low, moderate and high fatigue levels), while stiffness increases at low level, remains almost constant at moderate level, and decreases at high level of fatigue (Fung, Wang et al. 2009)

- On the other hand, changes in stiffness and hysteresis do not follow a monotonic pattern (Figure 2-13 and Figure 2-14)
- Stiffness increased and hysteresis decreased at low and moderate fatigue levels.
- Only at high-level fatigue, changes in stiffness and hysteresis were consistent to damaged tendon properties. (Figure 2-13 and Figure 2-14)

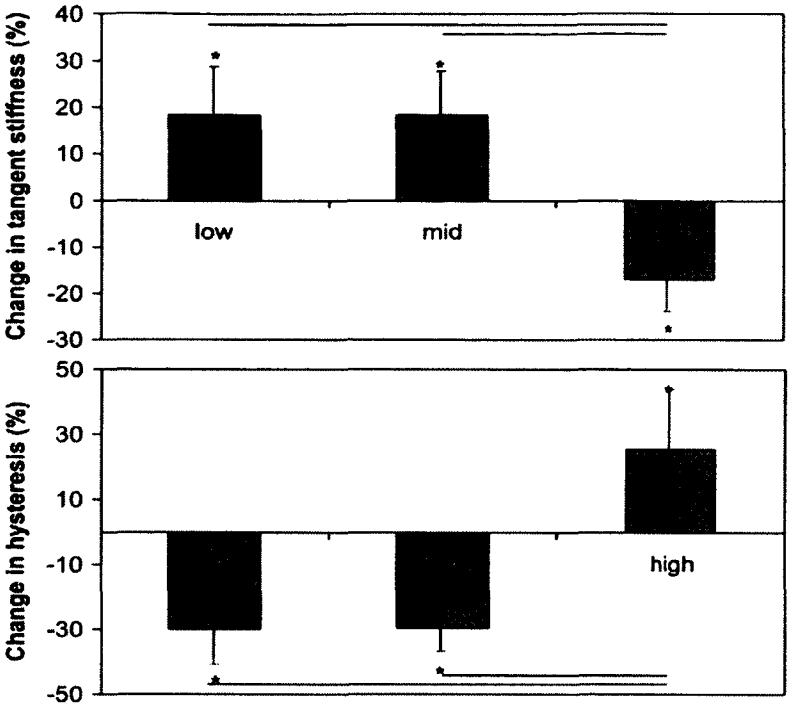

Figure 2-14: changes in stiffness and hysteresis do not show a monotonic manner. Only at high-fatigue level their changes are consistent to expected changes in damaged tendon (Fung, Wang et al. 2009).

- Structural characterization and compositional characterization revealed the degradation in tendon:
  - ➢ Microscopy images showed collagen structure damage and disorganization increasing with increasing damage.
  - ➢ Collagen I, III, V mRNA expressions altered at all fatigue levels.
- Results are inconsistent with healing response of the lacerated tendon.

Article 2. Coordinate regulation of IL-1 ? and MMP-13 in rat tendons following subrupture fatigue damage. (Sun, Li et al. 2008)

Hypothesis/Objective:

Overloaded tendon induces a tendinopathy and alters gene expressions in a load-dependent manner.

Table 2-11: Summary of characterization tests conducted in this article

Mechanical characterization		**Structural characterization**		**Compositional characterization**	
Test	**Measured variable**	**Test**	**Analysis**	**Test**	**Analysis**
In-line Non-destructive Cycling Force controlled	Peak to peak strain	Destructive Microscopy (confocal microscopy)	Qualification	Destructive	Reverse transcription PCR (mRNA analysis of MMP-13, and IL-1?) Western blot (protein analysis of MMP-13, and IL-1 ?)

Result:

- The results demonstrated that tendon structural damage and changes in gene expression of MMP-13, and IL-1 ? are distinctly different between low- and moderate- level fatigue loadings.

Article 3. Exposure dependent increases in IL-1β, Substance P, CTGF and tendinosis in flexor digitorum tendons with upper extremity repetitive strain injury. (Fedorczyk, Barr et al. 2010)

Hypothesis/Objective:

I. Tendons undergo inflammation earlier than other degenerative changes, when it is subjected to high repetitive high force (HRHF) tasks.

II. Both responses of tendons, i.e. inflammation and degenerative changes, are exposure-dependent, i.e. the longer the HRHF task and the higher demand tasks, the greater tissue response.

III. Inflammatory and neurochemical changes in tendons are related to declines in grip strength.

Table 2-12: Summary of characterization tests conducted in this article

Mechanical characterization		**Structural characterization**		**Compositional characterization**	
Test	**Measured variable**	**Test**	**Analysis**	**Test**	**Analysis**
In-line Non-destructive Cycling Force-controlled	Grip strength (MPF[16])	Destructive Microscopy (OM)	Qualification Semi-Quantification (modified Bonar scale method)	Destructive	ELISA (measuring IL-1β) Immunohistochemistry Quantification (changes in IL-1 β, and substance P)

[16] Maximum pulling force

Result:

- Mechanical characteristic of tendon, represented by grip strength, underwent an early decrease.
- At the same time as change in grip strength, there was an increase in substance P, and IL-1 ⍰ cells.
- There was a later increase in macrophages, neutrophils, connective tissue growth factor (CTGF), and periostin like factor (PLF) fibroblasts.
- Structural changes occurred at the time of increasing macrophages, neutrophils, CTGF, and PLF fibroblasts.
- It is suggested that the early increase of IL-1⍰, which plays a role in initiating fibroblast proliferation and degenerative tendon changes, caused later degenerative changes.

2.9 Summary and concluding remarks

There are several techniques to characterize tissue quality. Among these techniques, some are destructive and others are non-destructive. Structural and compositional characterizations are usually referred to as destructive (e.g. OM, TEM, analysis of tissue protein content) although there are also some non-destructive techniques (e.g. culture media analysis). For mechanical characterization, there are also destructive tests (e.g. failure test) and non-destructive tests (e.g. low amplitude stress relaxation or creep tests).

All these characterization tests provide the complementary information to better understand tissue quality since compositional, structural and mechanical properties are interrelated. In fact, by performing more of these characterization tests, more aspects of tissue quality will be clarified.

In our view, in-line non-destructive tests should be prioritized over other mechanical characterization methods. In-line tests enable the monitoring of tissue progression

during experiments. Therefore, for experiments investigating tissue properties at regular time intervals, information at each time point could be extracted from the same sample, and there is no need to replicate the experiment at each time point using different samples. Moreover, since the stimulation and characterization are performed in the same apparatus, the risk of contamination, damage, and also the risk of reference loss (e.g. loss of initial tissue length) resulted from transferring the sample from one apparatus to another is eliminated (Mathieu Viens, Guillaume Chauvette et al. 2011).

Non-destructive tests during the experiments provide the opportunity to apply other compositional and structural analysis or failure test at the end of the experiment since the tissue is not damaged. Therefore, complementary information can be obtained. In addition, in non-destructive tests samples can be self-compared, thus, data accuracy is increased. Moreover, the number of samples and animals is decreased. However, it is worth noting that very few studies perform non-destructive tests, as destructive tests can provide some information which could not be achieved in non-destructive tests like load-to-failure and strain-at-failure.

Although in-line non-destructive tests can be beneficial to gather information, on all aspects of tissue quality, there are still concerns about whether these tests will affect or not the experimental results. In fact, even if they are conducted using physiological parameters, they could affect tissue mechanobiological response. Up until now, this domain has been poorly investigated. In fact, we did not find studies performed on this topic.

One factor which could have an effect on experimental results is using diagnostic tests instead of stimulation protocols to provide information for evaluating tissue properties. We investigated this factor in this Master's project. Using diagnostic tests has the advantage of defining a "reference standard" to evaluate tissue properties in all experiments. Therefore, the data between different experiments could be compared more reliably. But as mentioned, there is a concern that behavior of the tissue could be affected by diagnostic tests. In Chapter 4, we demonstrate that the diagnostic test

conducted to observe tissue quality at regular time intervals has an effect on tissue progression over time.

This arises from viscoelasticity and viscoplasticity behaviors of tissue. In chapter 3 (review article) this subject will be discussed with a in-depth look into it.

3. Viscoelasticity and viscoplasticity of fibrous load-bearing tissues influence tissue mechanobiological response

3.1 Avant-propos

Auteurs: Leila Jafari et Eve Langelier

Affiliation: PERSEUS Research Group, Mechanical Engineering Department, Université de Sherbrooke, Sherbrooke, Québec, Canada

Date de soumission: 4 April 2012

Revue: Connective Tissue Research

Titre en francais : La viscoplasticité et la viscoélasticité des tissus supportant des chargements influencent la réponse mécanobiologique tissulaire

Résumé français :

Même si les blessures affectant les tissus fibreux supportant des chargements, comme les tendons, ligaments, capsules et fascias, sont fréquentes, il n'y a actuellement aucun traitement qui résulte en une guérison optimale de ces tissus. La mécanobiologie, ce domaine de recherche qui examine la réponse des tissus vivants aux stimuli mécaniques, serait la cause de plusieurs de ces blessures et pourrait contribuer significativement au développement de stratégies pour une prévention et une guérison optimales. Toutefois, la littérature ne comporte pas encore de description de la façon dont la réponse des tissus aux stimuli biophysiques est affectée par la viscoélasticité et la viscoplasticité, deux comportements clés des tissus fibreux supportant des charges. Le principal objectif de cette revue est d'expliquer ces comportements, ainsi que leurs effets sur la réponse des tissus aux stimuli mécaniques, puisque ces concepts doivent être compris et considérés par les chercheurs dans leur quête vers des traitements optimaux basés sur la mécanobiologie. Dans cet article, nous faisons une revue des connaissances et des hypothèses actuelles expliquant l'origine de la viscoélasticité et de la viscoplasticité dans les tissus fibreux supportant des chargements. Nous décrivons la

dynamique compétitive entre la réparation, la dégradation enzymatique et la dégradation mécanique, qui dépendent de la qualité du tissu, de même que de la viscoélasticité et la viscoplasticité. Finalement, nous présentons différents paramètres de stimulation qui influencent la réponse des tissus vivants supportant des chargements aux stimuli mécaniques à cause de leur viscoélasticité et de leur viscoplasticité. Cette analyse pourrait avoir des implications significatives pour les études *in vitro* en bioréacteur sur la pathophysiologie et le génie tissulaire fonctionnel, de même que pour les applications cliniques *in vivo*.

Title: Viscoelasticity and viscoplasticity of fibrous load-bearing tissues influence tissue mechanobiological response

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Running Title: Viscoelasticity and viscoplasticity in tissue mechanobiology

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3.2 Abstract

Although injuries occur frequently in fibrous load-bearing tissues, such as tendons, ligaments, capsules and fasciae, there are currently no treatments that result in optimal healing. Mechanobiology, the field of research into the response of live tissues to biophysical stimuli, lies behind many of these injuries and may potentially provide a significant contribution to the development of optimal prevention and healing strategies. However, the literature does not yet contain descriptions of how tissue response to biophysical stimuli is affected by viscoelasticity and viscoplasticity, two key behaviors of fibrous load-bearing tissues. The main objective of this review is to explain these behaviours, as well as their effects on tissue response to mechanical stimuli, since these concepts must be understood and accounted for by researchers in their quest for optimal treatments based on mechanobiology. In this paper, we review the current knowledge and hypothesis behind the origins of viscoelasticity and viscoplasticity in fibrous load-bearing tissues. We describe the dynamic competition between repair, enzymatic degradation and mechanical degradation of the extracellular matrix, which depends on tissue quality, as well as tissue viscoelasticity and viscoplasticity. Finally, we present different stimulation parameters influencing the response of live fibrous load-bearing tissues to biophysical stimuli because of viscoelasticity and viscoplasticity. This analysis may prove to have significant implications for bioreactor studies involving pathophysiology and functional tissue engineering, as well as for *in vivo* clinical applications.

Key words: Tendon, Ligament, Extra-cellular matrix, Stress relaxation, Creep, Mechanotransduction, Biophysical stimuli, Repair, Mechanical degradation, Enzymatic degradation, Tissue adaptation, Bioreactor, Rehabilitation, Tissue engineering

3.3 Introduction

Lesions to fibrous load-bearing tissues (FLBT), such as tendons, ligaments, capsules or fasciae, occur frequently in sports and occupational activities. Despite a high incidence of lesions, there are as yet no optimal treatments. Therefore, promoting native tissue healing, optimizing rehabilitation procedures and improving engineered tissues all constitute significant issues. To this end, researchers currently harbour high expectations of strategies based on mechanobiology.

Mechanobiology is the field emerging from mechanics and biology in studying how live tissues are produced, maintained and adapted by cells in response to biophysical stimuli (van der Meulen and Huiskes 2002). Mechanotransduction is at the heart of mechanobiology; it is the process of converting biophysical stimuli into biochemical signals at the cellular level (Wang 2006). It enables cells to "sense" applied biophysical stimuli (Wang 2006).

The response of FLBT to biophysical stimuli is governed, in part, by tissue viscoelasticity and viscoplasticity. Because of these two macro-mechanical behaviours, the type of stimulus (strain- or stress-controlled stimulus), the stimulus history and the use of recurring non-destructive mechanical tests for tissue quality characterization can influence tissue response. Thus, when dealing with FLBT response to biophysical stimuli, researchers cannot overemphasize the importance of considering tissue's viscoelasticity and viscoplasticity. They must understand and consider these macro-mechanical behaviours when selecting the most optimal stimulus and designing exercise-based treatment strategies.

The goal of this review is to introduce the inter-disciplinary community of researchers interested in FLBT mechanobiology (biologists, engineers, orthopaedists, etc.) to the impacts of viscoplasticity and viscoelasticity on tissue response to biophysical stimuli. First, we propose to briefly summarize the composition and structure of FLBT and then to review the current knowledge and assumptions explaining the origin of viscoelasticity and viscoplasticity in FLBT. Using a block diagram representation, we will deconstruct the live tissue response to biophysical stimuli into the components of

extracellular matrix (ECM) response and live cell response. This will lead to an explanation of the dynamic competition between the repair and degradation (mechanical and enzymatic) of the ECM, which in turn depends on tissue quality, viscoelasticity and viscoplasticity. Finally, we will discuss the influence of the aforementioned tissue mechanical behaviours on *in vitro* bioreactor studies and *in vivo* clinical applications. The explanations contained herein are based on the scientific literature.

Please note that this paper is aimed at an inter-disciplinary community of researchers. Therefore, a number of technical words or expressions used throughout this paper are defined in the Appendix to facilitate comprehension by readers from all research and practice backgrounds.

3.4 The composition and structure of FLBT

Throughout this paper, FLBT will be regarded as involving two main components: 1) an inert component made up of the ECM and 2) an active component constituted by the cells. FLBT are relatively hypocellular, as cells occupy only approximately 20% of the volume of tissue in tendons and ligaments (Nordin and Frankel 2001);(Oatis 2009). Therefore, it is reasonable to assume that the ECM provides the tissue's macro-mechanical behaviour.

The ECM contains approximately 60%-80% water and 20%-40% solids (Nordin and Frankel 2001) but these quantities vary with species (Vogel 1991), anatomical site (Amiel, Frank et al. 1984; Nordin and Frankel 2001; Keyoung Jin Chun 2003; Wang 2006), tissue types (Amiel, Frank et al. 1984) and age (Elliott 1965; Kleiner 1998; Nordin and Frankel 2001), *inter alia.* The ECM is a biopolymer, i.e. an interlaced network composed of three main categories of molecules: collagen, elastin and ground substance. (Please note that describing these molecules goes beyond the scope of this review. To that end, the reader may refer to the many available books and review articles.) The interactions among molecules of the same type, molecules of different types, as well as their interactions with water, dictate FLBT macro-mechanical behaviour.

3.5 Viscoelasticity and viscoplasticity of FLBT: Origin and manifestations

In order to study FLBT mechanobiological response, tissues are subjected to macroscopic biophysical stimuli. Under these biophysical stimuli, FLBT exhibit viscoelasticity or viscoplasticity. This section explains how the composition and structure of FLBT are at the heart of viscoelasticity and viscoplasticity. The manifestations of these macro-mechanical behaviours are also examined here.

3.5.1 Knowledge and assumptions behind the origin of FLBT macro-mechanical behaviour

Given the combination of biopolymers and water described above, the ECM in FLBT shows time-dependent (viscous) macro-mechanical behaviour with reversible (elastic) or non-reversible (plastic) deformation under biophysical stimuli, depending on the situation. Explanations proposed for these important behaviours are as follows:

Viscosity in the ECM could originate in part from the proposed mechanism of frictional losses related to fluid flow through the ECM (Sander and Nauman 2003). Another possible mechanism is frictional losses associated with the relative motion of ECM collagen structures as they pass by each other (Sander and Nauman 2003; Screen 2008). Because of viscosity, ECM mechanical behaviour is time-dependent, meaning that it is affected by loading rate and loading history. In FLBT, viscosity is combined with elasticity or plasticity:

Elasticity of the ECM could arise mainly from reversible extension of collagen units. First, at very low levels of loading, collagen fibres lose their waviness through the processes of straightening or uncrimping (Viidik 1972; Hansen, Weiss et al. 2002). Then, with increased loads, collagen helices (tropocollagens) begin to stretch (Mosler, Folkhard et al. 1985). Sliding of collagen units past each other is another mechanism explaining FLBT extension (Screen HR 2004). Since the uncrimping of collagen fibres and the stretching of helices are reversible processes (Józsa LG 1997), and since the

sliding of collagen units can be completely reversible under certain conditions (Screen HR 2004), the tissue shows viscoelastic behaviour.

Plasticity of the ECM occurs at higher levels of loading, for example, during a rupture test *in vitro* (Screen HR 2004; Oatis 2009)and with *in vivo* trauma (Nordin and Frankel 2001; Magee, Zachazewski et al. 2007). It also occurs after a higher number of lower loading repetitions, for example during an in vitro fatigue test (Wang, Ker et al. 1995; Wren, Lindsey et al. 2003; Thornton GM 2007; Fung, Wang et al. 2009; Fung, Wang et al. 2010; Parent G 2011), in vivo stretching (Kisner C 2007), and possibly during the development of overuse injuries (Magee, Zachazewski et al. 2007; Woo SL-Y 2007). This phenomenon could be explained through a non-reversible process of sliding between collagen units (Knörzer E 1986), a decrease in the amount of intramolecular bonds or through micro-damage to the ECM (Fung, Wang et al. 2009; Parent G 2011). The FLBT are then said to be viscoplastic.

Viscoelasticity and viscoplasticity both depend on variables relating to ECM quality, such as hierarchical structure, water content, noncollagenous ECM component content, and enzymatic and nonenzymatic cross-linking:

Hierarchical structure: A study by Gupta *et al.* (Gupta, Seto et al. 2010)showed that changes in tendon viscoelastic behaviour correlates with structural changes at the fibre and fibril levels.

Water content: Studies observed that tissue strength reduces with an increased hydration in tendon fascicles (Screen, Shelton et al. 2005; Screen, Chhaya et al. 2006)and that creep (time-dependent strain under stress; Figure 3-1) decreases with a decreased hydration in ligaments (Thornton, Shrive et al. 2001).

Noncollagenous ECM component content: Using decorin knockout mice, larger and faster stress relaxations (time-dependent stress under strain; figure 3-1) were observed in the absence of decorin (Elliott, Robinson et al. 2003). Moreover, tendon fascicles from which glycosaminoglycans were removed using the enzyme

chondroitinase ABC exhibited an increased maximum modulus, compared with freshly extracted fascicles (Screen, Chhaya et al. 2006).

Cross-linking: It is recognized that collagen cross-linking prevents molecule sliding and increases tissue stiffness (DeGroot 2004; Avery and Bailey 2005).

Finally, it is worth noting that, over a lifetime, changes in the viscoelasticity and viscoplasticity of FLBT occur, depending on various life stages or events, such as maturation (Lam, Frank et al. 1993), ageing (Nielsen, Skalicky et al. 1998), injury (Dourte, Perry et al. 2010), healing (Frank, Hart et al. 1999; Abramowitch, Woo et al. 2004) or immobilization (Eliasson, Fahlgren et al. 2007), all of which affect the ECM quality (Józsa LG 1997).

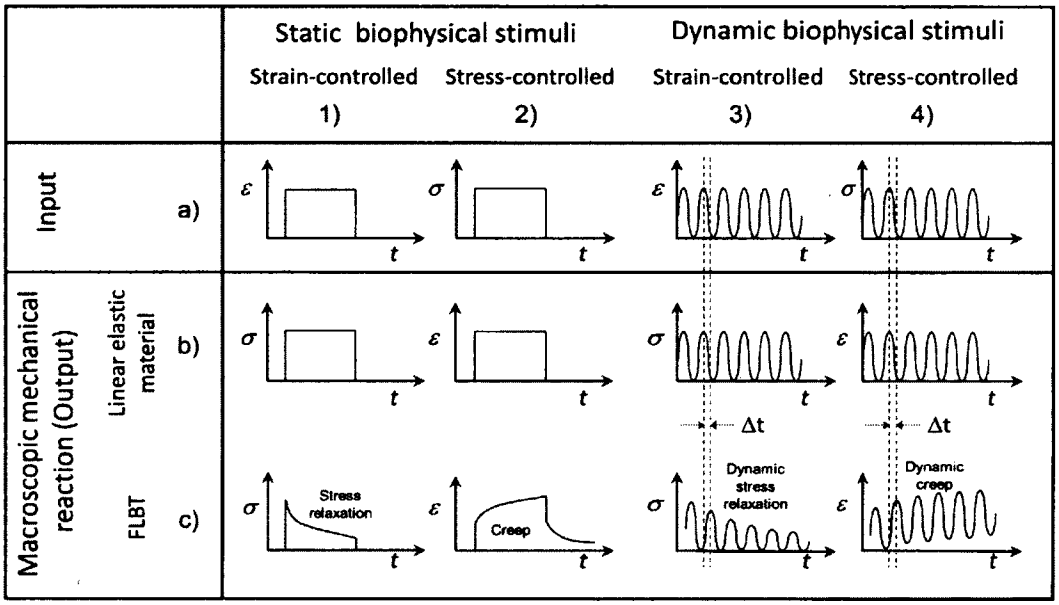

Figure 3-1: Comparison of the manifestations of linear elasticity of materials and viscoelasticity/viscoplasticity of FLBT under static and dynamic stimuli. ε is strain; σ is stress; t is time and Δt is time delay

3.5.2 Manifestations of viscoelasticity and viscoplasticity for ECM under biophysical stimuli

In order to appreciate the impact of the ECM's macro-mechanical behaviour on the mechanobiological response of live tissues, we must first highlight the differences between the manifestations of elasticity, viscoelasticity and viscoplasticity of inert materials (tissues without live cells) when they are submitted to strain- or stress-controlled biophysical stimuli.

Materials react differently to loading, depending on their macro-mechanical behaviour, as shown in Figure 3-1. A linear elastic material follows Hook's law: $\sigma = E\varepsilon$, where σ is stress, E is Young's modulus and ε is strain. Thus, the ratio σ/ε is constant over time with both static and dynamic stimuli (Figure 3-1, 1: 1a vs 1b; 2a vs 2b; 3a vs 3b; 4a vs 4b).

A viscoelastic material shows a time-dependent reaction to load. As a consequence, when subjected to constant strain input, the resulting output is stress relaxation ($\varepsilon = f(\sigma,t)$) (Figure 3-1: 1a vs 1c) and when subjected to constant stress the output is creep ($\sigma=f(\varepsilon,t)$) (Figure 3-1: 2a vs 2c). *In vivo*, stress relaxation and creep can be experienced during static stretching. Stress relaxation occurs for example when one keeps a stretching position constant and feels the stretching sensation decreasing with time. Creep occurs when a stretching force is maintained constant by adjusting one's position over time.

When subjected to dynamic stimuli, the ECM of FLBT shows a time delay between stimulation and response (Figure 3-1: 3a vs 3c; 4a vs 4c). Moreover, because of time-dependence, the output resulting from dynamic strain input is dynamic stress relaxation (Figure 3-1: 3a vs 3c) and the output from dynamic stress input is dynamic creep (Figure 3-1: 4a vs 4c).

Dynamic stress relaxation (Figure 3-1: 3c) and dynamic creep (Figure 3-1: 4c) can be explained by incomplete recovery of the tissue's mechanical properties between two

successive loading cycles. Incomplete recovery, in turn, can be explained by two mechanisms:

1) The viscous phenomena have different time constants during tissue loading and unloading phases. For example, in figure 3-2, tissue lengthening is driven by the apparatus during the loading phase of a strain-controlled stimulus. However, during the unloading phase, tissue shortening is driven by the tissue itself. This may require more time than allowed by the stimulation protocol.

2) During loading, the tissue's quality changes (temporarily or permanently) as water is exuded, collagen fibrils slide against each other, the collagen structure is damaged, proteoglycans are lost, etc. These changes in tissue quality imply that changes (temporary or permanent) occur in mechanical properties and in the time constant for viscous phenomena.

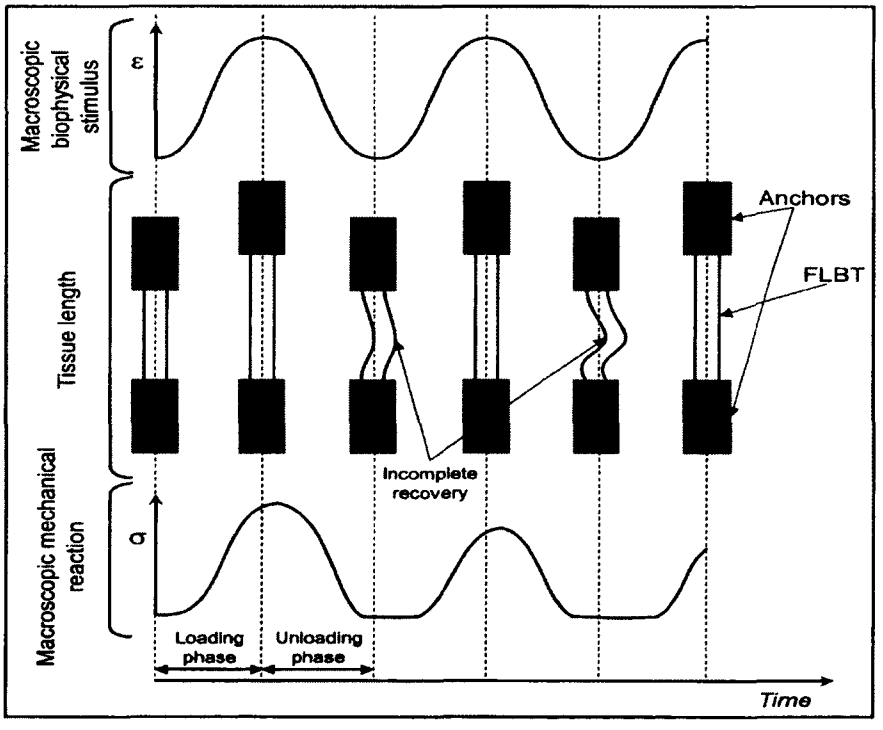

Figure 3-2: Dynamic stress relaxation during a strain-controlled dynamic test. Incomplete recovery of tissue length and mechanical properties at the end of the unloading phase leads to reduced peak stress at the end of the next loading phase. (Please note that changes were emphasized in the figure to facilitate conceptualization. However, in reality, changes may be more subtle, as they may occur microscopically, such as in molecular rearrangement).

Alternating stimuli/rest periods also highlight a difference between the behaviours of elastic materials and viscoelastic/viscoplastic FLBT (Figure 3-3). For example, periods of dynamic loading under strain control can be alternated with rest periods. In FBLT, stress level decreases during stimulation periods and recovers during rest periods (Figure 3-3a). However, if the rest periods are too short (Solomonow, He Zhou et al. 2000; Solomonow 2004)(Figure 3-3b-c) or if plasticity has occurred in the ECM, stress recovery may be hindered.

Finally, it is worth noting that both static and dynamic creep can lead to rupture if maintained over a long enough period (Wang and Ker 1995; Wang, Ker et al. 1995; Wren, Lindsey et al. 2003; Thornton GM 2007; Fung, Wang et al. 2009; Parent G 2011). Dynamic creep apparently increases faster than static creep (Thornton, Shrive et al. 2001).

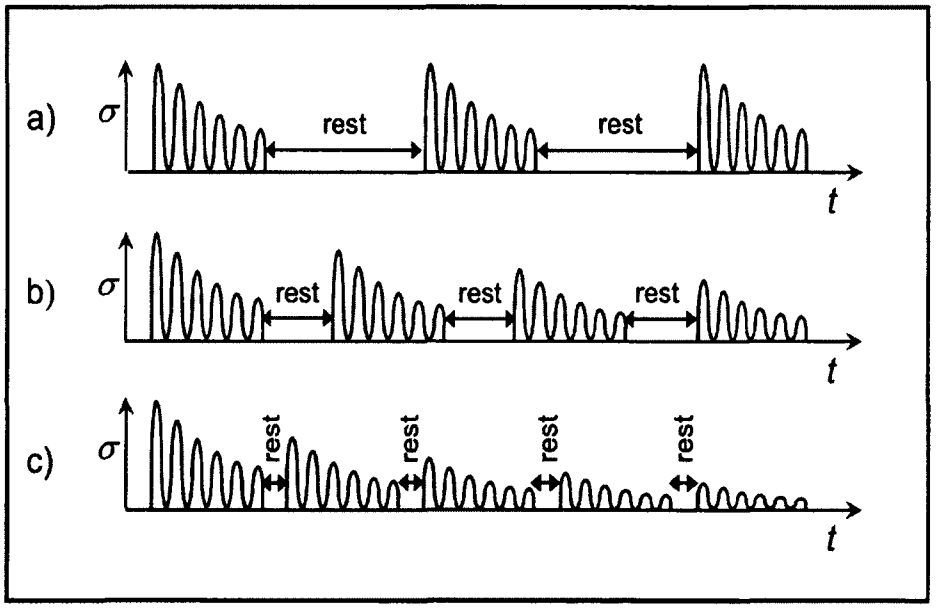

Figure 3-3: Impact of rest periods on the manifestation of material viscoelasticity/viscoplasticity under strain-controlled dynamic stimuli. When rest periods are too short (b and c), the overall stress level experienced by the ECM decreases. σ is stress and t is time. Double-headed arrows indicate rest periods. (Adapted from Viens *et al.* (2011) *ASME Journal of Medical Device* with permission).

3.6 Live FLBT response to biophysical stimuli

The global response of live FLBT to biophysical stimuli is complex. To facilitate understanding, the following sections deconstruct the tissue response into the separate components of ECM micro-mechanical and cellular responses, and then bring them back together in order to address the global tissue response.

3.6.1 ECM response under biophysical stimuli

Under macroscopic biophysical stimuli, the molecules that make up the inert ECM are subjected to stress and strain and could thus undergo mechanical degradation (MD), as would many conventional polymers under similar mechanical loading conditions (Ward 1983) (Figure 3-4 in blue). One example of MD is mechanical fatigue, which affects tissue quality X over time (and thus implies a time rate of change of tissue quality $\dot{X}_{MD}$) when the ECM is submitted to dynamic biophysical stimuli. LBFT fatigue can occur due to repetitive activities at work or in sports. Another example of MD is partial or complete rupture of the ECM when it is subjected to excessive stress, such as in trauma. ECM plasticity is a manifestation of MD due to microstructural changes in the ECM and its components (Fung, Wang et al. 2009; Parent G 2011).

Research on the MD process affecting the ECM under stress-controlled dynamic stimuli has generally shown that the ECM strain adopts a triphasic shape over time since strain always increases but at different rates over time (Wang, Ker et al. 1995; Wren, Lindsey et al. 2003; Thornton GM 2007; Fung, Wang et al. 2009; Fung, Wang et al. 2010; Parent G 2011) (Figure 3-5 a). Compliance (the inverse of stiffness) changes in a U-shaped curve over time (Parent G 2011), meaning that compliance first decreases and later increases over time (Figure 3-5 b). Depending on the study, stiffness either decreases over time (Wang, Ker et al. 1995; Wren, Lindsey et al. 2003), or changes in an inverse-U curve (Fung, Wang et al. 2009; Fung, Wang et al. 2010) meaning that stiffness first increases and later decreases over time (Figure 3-5 b). This corresponds to the compliance observations. Both the time rate of change of strain (Wang, Ker et al. 1995;

Wren, Lindsey et al. 2003; Fung, Wang et al. 2009; Fung, Wang et al. 2010; Parent G 2011) and

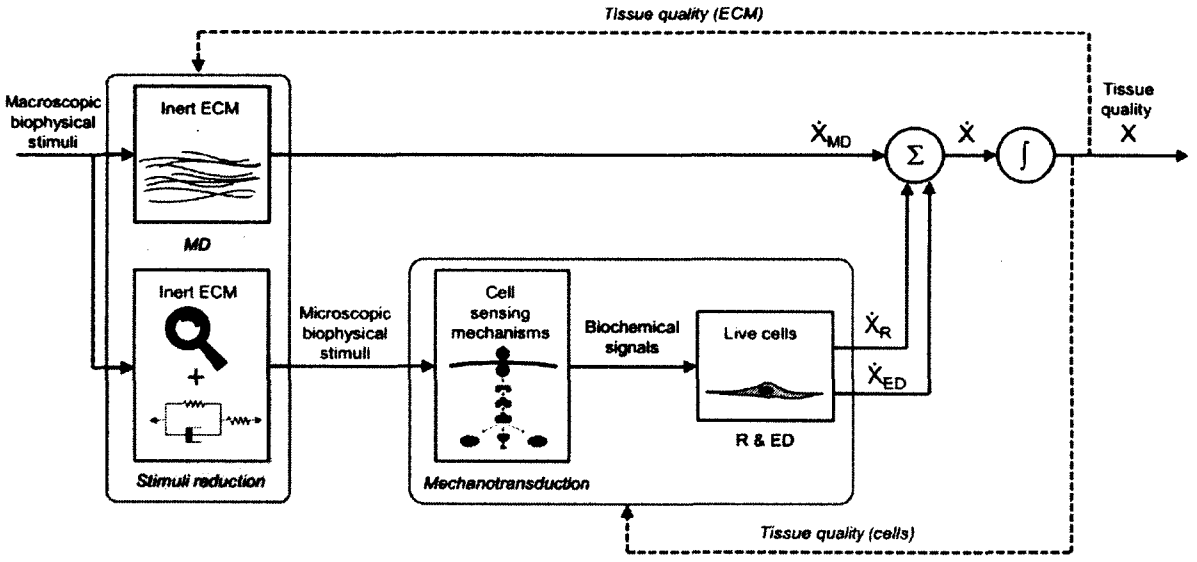

Figure 3-4: Block diagram representation of the mechanobiological response of FLBT under biophysical stimuli including the impact of viscoelasticity/viscoplasticity. In blue: Under macroscopic biophysical stimuli, the inert extracellular matrix (ECM) undergoes mechanical degradation (MD) which affects the time rate of change of tissue quality ($\dot{X}$). In green: The ECM reduces the macroscopic stimuli applied to the tissue as a whole into microscopic stimuli detected by the cells. This process is called mechanotransduction. The resulting biochemical signals instruct the cells to repair (R) or use enzymatic degradation (ED) on the ECM, which again affects $\dot{X}$. In red: As the tissue progresses in response to stimuli, its quality X changes. Thus, the microscopic stimuli, biochemical signals, R, ED and MD also progress, as illustrated by the tissue quality feedback. In orange: Because of viscoelasticity and viscoplasticity, the microscopic stimuli sensed by the cells change over time, even though the macroscopic biophysical stimuli remain constant. The spring and dashpot model used to represent these macro-mechanical behaviours in the block diagram refers to the widely used Zener model in linear viscoelasticity.

the time rates of change of compliance and stiffness (Wang, Ker et al. 1995; Wren, Lindsey et al. 2003; Fung, Wang et al. 2009; Fung, Wang et al. 2010; Parent G 2011) accelerate before rupture. Under strain-controlled stimuli, however, the peak stress decreases nonlinearly over time (Figure 3-5 c)(Cousineau-Pelletier P 2009).

From a micro-structural point of view, the ECM of tendons (without cellular activity) subjected to stress-controlled dynamic stimuli exhibit histological alterations in the collagen network, increasing nonlinearly with fatigue levels (Elliott, Robinson et al. 2003; Wren, Lindsey et al. 2003; Parent G 2011), and developing in a non-uniform

fashion over the tendon (Fung, Wang et al. 2009; Parent G 2011). From a macro-structural point of view, tendon diameter increases with damage (Lanir, Salant et al. 1988).

3.6.2 Cellular response to biophysical stimuli

The pathway relating macroscopic biophysical stimuli to cellular response is illustrated in Figures 3-4 (in green) and 3-A-1. First, macroscopic stimuli on the tissue are reduced to microscopic stimuli detected by the cells, as illustrated by the magnifying glass. The ECM acts as a transfer function converting macroscopic biophysical stimuli into microscopic stimuli: fluid flow (Butler, Kohles et al. 1997; Sander and Nauman 2003), ion and molecule movements and gradients (Grodzinsky 1983), pressure gradients (Haemer, Carter et al. 2012), as well as stress and strain in the ECM which affect the individual cells (Matyas, Edwards et al. 1994; Arnoczky, Lavagnino et al. 2002; Screen HRC 2003; Upton, Gilchrist et al. 2008; Gupta, Seto et al. 2010; Lai and Levenston 2010). Then, these microscopic stimuli are sensed by the cells through different mechanisms including membrane proteins, the cytoskeleton, stretch activated channels, and primary cilia as presented in different review articles (Wang 2006; Janmey and McCulloch 2007; Wang, Thampatty et al. 2007; Wang N 2009). These mechanisms transform the microscopic stimuli into biochemical signals that are detected by the cells, a process called "mechanotransduction". In response to these biochemical signals, the live cells can react through repair (R) of the ECM via the secretion and assembly of molecules such as collagen (Kjaer, Magnusson et al. 2006; Devkota, Tsuzaki et al. 2007; Kjaer, Langberg et al. 2009), or enzymatic degradation (ED) of the ECM by means of production and activation of proteases such as matrix metalloproteinases (MMPs) (Arnoczky SP 2007; Devkota, Tsuzaki et al. 2007; Cousineau-Pelletier P 2009). Moreover, if the magnitude and duration of the microscopic stimuli are too high, the cells may undergo apoptosis, a programmed cell death, as a result of activation of intracellular stress-activated protein kinases (Yuan, Wang et al. 2003). If the magnitude and duration of the microscopic stimuli are too low, cells may also undergo apoptosis (Woo SL-Y 2007).

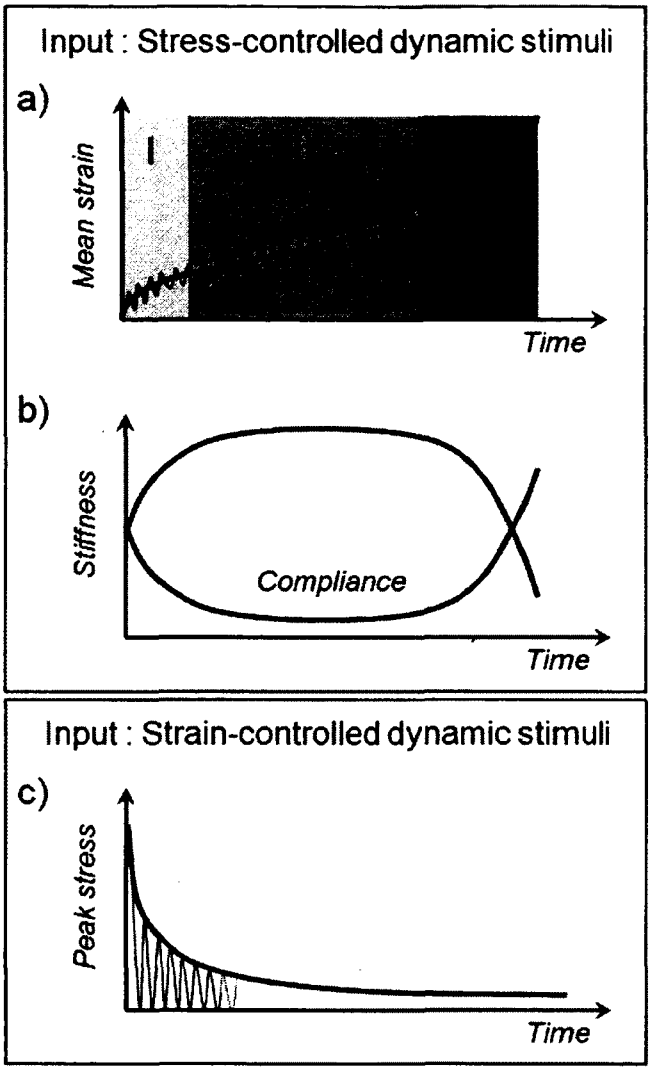

Figure 3-5 : Manifestation of material viscoelasticity/viscoplasticity under dynamic stimuli. (A) Under stress-controlled stimuli, mean strain follow a triphasic pattern (A), compliance follows a U curve (B) while stiffness follows a U-inverse curve (B). Under strain-controlled stimuli, the peak stress decreases nonlinearly over time (C).

With regard to the process of ED, studies have shown an increase in matrix metalloproteinase (MMP) production under stress deprivation (Arnoczky, Lavagnino et al. 2007; Arnoczky, Lavagnino et al. 2008; Gardner, Arnoczky et al. 2008). The same outcome can be observed under dynamic stimuli, but in a magnitude- and duration-dependent manner (Devkota, Tsuzaki et al. 2007). Moreover, tendinopathic tendons exhibit MMP up-regulation. Finally, in the R process, studies have shown that collagen

and glycosaminoglycan productions are induced by suitable mechanical stimulation (Screen, Shelton et al. 2005; Kjaer, Magnusson et al. 2006; Devkota, Tsuzaki et al. 2007; Maeda, Shelton et al. 2007; Kjaer, Langberg et al. 2009; de Almeida, Tomiosso et al. 2010).

3.6.3 Global FLBT response to biophysical stimuli

The global FLBT response to biophysical stimuli combines the ECM response (Figure 3-4 in blue) and the cellular response (Figure 3-4 in green). (Please note that Figure 3-A-1 in the Appendix also shows global FLBT mechanobiological response but without using the block diagram representation). The competitive dynamics of MD, ED and R regulate tissue mechanobiological response (TMR), which can be expressed, as a first approximation, as: $TMR = R - MD - ED$ where $\dot{X}_R \neq \dot{X}_{MD} \neq \dot{X}_{ED}$. These differences in repair and degradation rates occur because, *inter alia*, protein expression takes time to occur. Moreover, repair and degradation rates vary in different ways according to the biophysical stimuli (amplitude, frequency, rest periods, etc.) and tissue quality. For example, immobilization increases $\dot{X}_{ED}$ but decreases $\dot{X}_R$ and $\dot{X}_{MD}$.

Thus, depending on the biophysical stimuli, TMR can lead to tissue homeostasis, improvement or degeneration in an inverse-U relationship (ScienceDirect 2011). The clinical implications are that immobilization (under-stimulation) results in tissue weakening (Woo, Gomez et al. 1982; Józsa LG 1997; Wang, Iosifidis et al. 2006; Woo, Abramowitch et al. 2006; Woo SL-Y 2007), while training (stimulation) results in improvement of tissue mechanical performance (Woo, Gomez et al. 1982; Wang, Iosifidis et al. 2006), and overtraining or overuse (over-stimulation) results in tissue damage (Józsa LG 1997; Wang, Iosifidis et al. 2006; Woo, Abramowitch et al. 2006; Woo SL-Y 2007).

As tissue degrades or improves in response to biophysical stimuli, the quality of the ECM is altered (Kannus and Jozsa 1991; Jarvinen, Jozsa et al. 1997; Józsa LG 1997; Cook JL 2004; Scott, Cook et al. 2007; Woo SL-Y 2007; Maffulli, Longo et al. 2008; Xu and Murrell 2008). Cell attributes, such as shape, phenotype and live/dead state, also vary

(Jarvinen, Jozsa et al. 1997; Józsa LG 1997; Cook JL 2004; Scott, Cook et al. 2007; Woo SL-Y 2007; Maffulli, Longo et al. 2008; Xu and Murrell 2008). Consequently, under constant macroscopic stimuli, the microscopic stimuli, biochemical signals, R, ED and MD will progress and change according to feedback from the tissue quality (Figure 3-4 in red).

These observations led Arnockzy *et al* (Arnoczky SP 2007)to propose a new hypothesis in tendinopathy: microtraumas to the collagen structure create an environment of mechanobiological under-stimulation, leading to pathological changes, such as increased MMP and apoptosis. In other words, as the collagen structure degrades, fewer microscopic stimuli are received by the individual cells. Consequently, cells eventually become under-stimulated and enter the left part of the Inverse-U curve, leading to further tissue degradation.

3.7 Impact of ECM viscoelasticity and viscoplasticity on live FLBT response to biophysical stimuli

The response of live FLBT to biophysical stimuli is affected by the viscoelastic/viscoplastic behaviours of the ECM (Figure 3-4 in orange). During the application of biophysical stimuli, we observe that the microscopic stimuli sensed by the cells change over time, even though the macroscopic biophysical stimuli remain constant. Static or dynamic stress relaxation and creep experienced by the ECM result in microscopic stimuli (e.g. fluid flow and cell deformation) that vary over time. Moreover, these stimuli progress differently under strain- or stress-controlled macroscopic stimuli (Figure 3-1). This necessarily has consequences for the cell's mechanobiological response.

The impact of viscoelasticity/viscoplasticity on the cellular response is a complex one. For example, under dynamic stress stimuli, the ECM undergoes dynamic creep, which could lead to micro-damage if the degradation rate ($\dot{X}_{MD} + \dot{X}_{ED}$) were to be greater than the repair rate ($\dot{X}_R$). As micro-damage to the ECM occurs, $\dot{X}_{MD}$ increases (Wang, Ker et al. 1995; Wren, Lindsey et al. 2003; Fung, Wang et al. 2009; Parent G 2011). Moreover, the

signals sent to the cells change, and if the cells become under-stimulated, $\dot{X}_{ED}$ increases (Lavagnino, Arnoczky et al. 2006; Arnoczky SP 2007). A "vicious circle" would emerge at this point. However, if rest periods were to be established between stimulation periods in order to allow the ECM to recover its initial length, and to provide time for the cells to repair the micro-damage, the outcome could be completely different.

Similarly, under dynamic strain stimulation, the ECM undergoes stress relaxation, which leads to cells becoming under-stimulated. Consequently, $\dot{X}_{ED}$ increases and, with a damaged structure, cell stimulation continues to decrease. Again, this potentially leads to a vicious circle. However, if the amplitude of the strain stimulation is increased regularly to maintain a minimum peak-to-peak stress amplitude, or if rest periods are included to allow for stress recovery, the outcome could once again be completely different.

Our group recently obtained experimental data to support this (Jafari ; Yoan Lemieux-LaNeuville 2012). The aim of our study was to investigate the effect of two characterization protocols on tissue mechanobiological alterations over time. We had two groups of live healthy tendons. We subjected the first group to mechanical stimuli at physiological amplitudes inside a bioreactor for 3 days. We subjected the second group to the same mechanical stimuli but also to 24 stress relaxation tests at physiological amplitudes each day. We compared alterations in each group over time and observed that stress relaxation tests at physiological amplitudes modified the tendon response to mechanical stimulation *in vitro*. These results are a demonstration of the effect of viscoelasticity/viscoplasticity of tissue on its response to mechanical stimuli.

3.8 Impact of ECM viscoelasticity and viscoplasticity on *in vitro* mechanobiological research and *in vivo* clinical applications

Knowing that viscoelasticity and viscoplasticity affect live FLBT response to biophysical stimuli, researchers must consider these macro-mechanical behaviours when designing experimental protocols for bioreactor studies or when attempting to improve clinical

applications. For *in vitro* studies, this translates into taking enlightened decisions about the following concerns:

- **Strain- or stress-controlled stimuli:** Many laboratories choose to work under strain- or displacement-control (Lavagnino, Arnoczky et al. 2003; Screen, Shelton et al. 2005; Maeda, Shelton et al. 2007; Cousineau-Pelletier P 2009), possibly because it is much simpler to implement in a bioreactor. Other laboratories implement stress- or force-control to mimic tendons which transmit forces from the muscle to the bone *in vivo* (Schechtman and Bader 1997; Ker, Wang et al. 2000; Yamamoto, Kogawa et al. 2005; Parent G 2011). This decision is not an easy one, especially as this subject has not been explored in depth. However, as explained earlier, it will have an impact on mechanobiological response because of tissue viscoelasticity and viscoplasticity and therefore needs to be considered.

- **Stimulus history:** Since FLBT contain live cells, but also because of their macro-mechanical behaviour, FLBT mechanobiological response is affected by rest periods. Preliminary testing can be easily done to explore the impact of rest periods on tissue response. However, it is important to keep in mind that as the tissue progresses over time, the impact of rest periods may also progress.

- **Intelligent bioreactors or adjustable protocols:** In response to macroscopic biophysical stimuli, tissue quality (of ECM and cells) changes. Consequently, the macro-mechanical behaviour and mechanobiological response also change. For optimal tissue improvement, macroscopic biophysical stimuli should be continually adjusted to tissue quality. This could be done manually or through intelligent bioreactors. However, once again this subject has not yet been thoroughly explored.

- **Mechanical characterization protocols:** Viscoelasticity and viscoplasticity could also have an impact on the characterization protocols used to evaluate tissue progression in bioreactor studies of FLBT response to biophysical stimuli. In such studies, characterization of tissue progression is essential to

understanding and eventually predicting its response to mechanical stimuli. There is, however, a paradox in measuring progression in live tissue: how can we accurately measure tissue progression over time if the tissue is also reacting to our measurement methods? (Jafari ; Yoan Lemieux-LaNeuville 2012)The methods used to observe tissue progression over time could introduce a bias because of the impact of stimulus history on ECM macroscopic behaviour (Jafari ; Yoan Lemieux-LaNeuville 2012).

With regard to clinical applications, tissue viscoelasticity and viscoplasticity are unavoidable concepts in the design and improvement of mechanobiology based treatment plans. For example, in injury prevention, it has been proposed that adequate rest between periods of physical activity is required to avoid ligament creep and its consequences such as joint laxity, instability and osteoarthritis (Solomonow, He Zhou et al. 2000; Solomonow 2004). In current rehabilitation practices, treatments using static creep and stress-relaxation are common. Examples include stretching to increase the range of motion (Kisner C 2007) or using orthotics over long periods to treat deformities such as scoliosis (Nordin and Frankel 2001). Treatments using dynamic stimulation also exist. Early mobilisation after tendon repair is one example. In that case, passive motion (i.e. without muscle contraction) to stimulate tissue repair and to avoid contracture caused by immobilisation (Kisner C 2007). Later, to minimize impairment of muscle performance, motion should progress from passive to active exercise in the following sequence: isometric, concentric, and finally eccentric movements (Kisner C 2007).

Another clinical application is the treatment of tendinosis, which can be very challenging. Studies have shown that approximately one-third of athletes with lower extremity tendinosis demonstrate poor outcomes with either conservative therapy or surgical treatment (Cook, Khan et al. 1997; Chiara Vulpiani, Guzzini et al. 2003). Tendon mechanical properties have been shown to change with strength training but more research is needed to shed light on the theoretical framework supporting the mechanotherapeutic effect of different types of exercise on tissue repair. For the past two decades, the preference of eccentric training over concentric training for the

conservative management of tendinopathy has been accepted, and whether eccentric exercise is more effective than other types of exercise to reduce symptoms or promote healing remains unresolved (Wasielewski NJ 2007). Therefore, we need to find a consensus on optimal parameters (duration, frequency, magnitude and type of mechanical stimulation) that should be applied to the tendon during the training program that will improve and/or accelerate the healing process without causing more damage to the tissue. This will be very challenging. For example, the maximum deformation and strain induced in tendons from in vivo muscle loading vary considerably according to variables such as age and sex, with values ranging from 2.5% to 10% (Maganaris and Paul 1999; Kubo, Kanehisa et al. 2001; Kubo, Kanehisa et al. 2003; Kubo, Kanehisa et al. 2003). This suggests that large inter-individual variations in tendon structural properties, joint mechanics and muscle-tendon-bone adaptation responses can be expected, and that a "one size fits all" treatment protocol may not be an option.

In order to improve clinical applications, we need a better understanding of *in vivo* biophysical stimuli induced by daily activities or exercises:

Muscle-tendon unit: For *in vivo situations*, we must consider the entire muscle-tendon unit and not solely the tendon as we do in *in vitro* bioreactor studies. The muscle, passive or contracted, has mechanical properties that affect the reaction of the whole tendon-muscle unit to biophysical stimuli. Moreover, the maximal force generated by a muscle is a function of its length and speed of contraction (Oatis 2009). Consequently, during exercises conducted at maximal muscle force, the load on the FLBT may vary with time. Studies to deepen our understanding of the tendon stimuli *in vivo* are therefore required. These studies should not only look at tendon stimuli over a few repetitions, but also over longer periods such as a work shift, since stimuli may change over this time period.

Strain- or stress-controlled stimuli: *In vivo*, the type of stress- or strain-control used the movements has not yet been clearly identified in real-life situations. Therefore,

making parallels between *in vivo* and *in vitro* situations is hazardous and should be assisted by theoretical and experimental investigations.

3.9 Concluding remarks and future perspectives

Despite progress in mechanobiology, there are still significant gaps in knowledge, in particular regarding the impact of: 1) the type of stimulus input (strain- or stress-controlled stimulus), 2) stimulus history, 3) changes in tissue quality, and 4) methods used to observe alterations in tissue over time. These topics have not been sufficiently investigated in bioreactor studies examining pathophysiology or functional tissue engineering, or even in *in vivo* clinical studies. Research in these areas is therefore required.

The authors' view is that dose-response experiments alone would not be sufficient to investigate these subjects because there are too many input possibilities (strain- or stress-controlled stimulus, amplitude, frequency, rest periods, tissue quality, etc.). Experiments could thus conceivably last for many years. Instead, we believe that theoretical models should first be created to relate macroscopic to microscopic stimuli for different tissue qualities. Then, well planned dose-response experiments should be conducted in conjunction with modelling, to identify the transfer functions between macroscopic stimuli, microscopic stimuli, ECM response and cellular response.

The theoretical model and experimental data together should facilitate an understanding of tissue mechanobiological response and allow prediction of the optimal stimulus to minimize $\dot{X}_{MD}$, and $\dot{X}_{ED}$ and maximize $\dot{X}_{R}$. For example, a combination of both types of stimulus input (strain- and stress-controlled stimuli), each used at different time points during FLBT rehabilitation, could be the best strategy to promote tissue healing, bearing in mind that $\dot{X}_{R}$, $\dot{X}_{MD}$, and $\dot{X}_{ED}$ all vary according to tissue quality, tissue viscoelasticity/viscoplasticity and stimulus input. The new knowledge could ultimately be used to improve *in vitro* and *in vivo* applications, such as functional engineered tissues, rehabilitation following tendon repair surgery, the ligamentization process following anterior cruciate ligament repair and the healing of tendinosis. For

clinical applications however, a better understanding of *in vivo* biophysical stimuli induced by daily activities or exercises is required, as pointed out earlier.

Key Points

1) Fibrous load-bearing tissues (FLBT) are well organized biopolymers containing a high proportion of extracellular matrix (ECM). Thus, the molecules network forming the ECM is responsible for the tissue macro-mechanical behaviours.

2) FLBT have viscoelastic or viscoplastic macro-mechanical behaviours. They exhibit reversible or non-reversible deformation accompanied by energy losses which depend on ECM quality variables, such as structure, composition and cross-linking.

3) Because of their viscoelastic or viscoplastic macro-mechanical behaviours, FLBT reaction to biophysical stimuli is influenced by the type of stimulus input (strain- or stress-controlled stimulus) and stimulus history (including rest periods).

4) Under macroscopic biophysical stimuli, ECM molecules are subjected to stress and strain, and could thus undergo mechanical degradation (MD). Changes to mechanical properties and ECM structure related to MD in FLBT are non-linear over time and heterogeneous across the tissue.

5) When FLBT are subjected to macroscopic biophysical stimuli, these stimuli are scaled down to microscopic biophysical stimuli which are, in turn, transformed into biochemical signals that the cells can sense. In response to these signals, the cell can degrade the ECM via enzymatic degradation (ED) or repair (R) it.

6) The tissue global mechanobiological response is the result of competitive dynamics between degradation and repair leading to an inverse-U relationship between stimulation and tissue quality. Moreover, the three processes of R, ED and MD involved in these dynamics are inter-related, since they all affect and depend on ECM and cell quality at the same time. They also have different time rates of action.

7) In conclusion, viscoelasticity and viscoplasticity of FLBT influence tissue mechanobiological response and must be considered when identifying the macroscopic biophysical stimuli to promote the healing of native tissues, to optimize rehabilitation after surgery or to improve engineered tissues.

Acknowledgments

This work was supported by NSERC Grant No. 299280 and a CIRUS scholarship awarded to Leila Jafari. We thank Saïd Elkoun for providing technical insights on materials and Jean-Marc Drouet for his critical review of the article.

Appendix

Below are the definitions the authors consider to be vital to understanding the ideas contained in this paper.

Tissue quality: Condition of a tissue (ex: healthy, damaged) determined by different parameters, such as composition, structure and cross-linking for the ECM, or viability and proliferation for cells.

Macroscopic biophysical stimuli: Static or dynamic mechanical loadings applied macroscopically to the tissue. Biophysical stimuli can be created *in vitro* inside a bioreactor, or *in vivo* through daily activities such as at work or during training.

Strain-controlled stimuli: Macroscopic biophysical stimuli applied under strain control, for example, a sinusoidal stimulus of 1% strain amplitude applied at 1Hz to the tissue.

Stress-controlled stimuli: Macroscopic biophysical stimuli applied under stress control, for example, a sinusoidal stimulus of 1MPa stress amplitude applied at 1Hz to the tissue.

Macro-mechanical behaviour: A macroscopic mechanical behaviour of a material, which in this context refers specifically to viscosity, elasticity and plasticity, or to their combinations (viscoelasticity and viscoplasticity).

Manifestation of ECM behaviour: A macroscopic mechanical reaction of the viscoelastic or viscoplastic ECM to biophysical stimuli. For linear elastic materials, for example, this reaction is governed by Hook's law. This means that stress and strain are linearly related via Young's modulus.

Microscopic biophysical stimuli: Biophysical stimuli inside the tissue are detected by the individual cells; this includes fluid flow, ion and molecule movements and gradients, pressure gradients and stress/strain in the molecules that compose the ECM. A microscopic biophysical stimulus is produced when a macroscopic biophysical stimulus is applied to the tissue.

ECM response: Microscopic mechanical reaction of the ECM to biophysical stimuli, which in this context refers to the mechanical degradation of the ECM

Mechanotransduction: The conversion of microscopic biophysical stimuli into biochemical signals. One example of mechanotransduction is the movement of ions through channels activated by the mechanical stretch of the cellular membrane.

Cellular response: The biological reaction of live cells to biophysical stimuli, which in this context refers to the repair and enzymatic degradation of the ECM.

Mechanobiological response: Global reaction of live tissues to biophysical stimuli. This includes ECM and cellular responses, and therefore mechanical degradation, repair and enzymatic degradation.

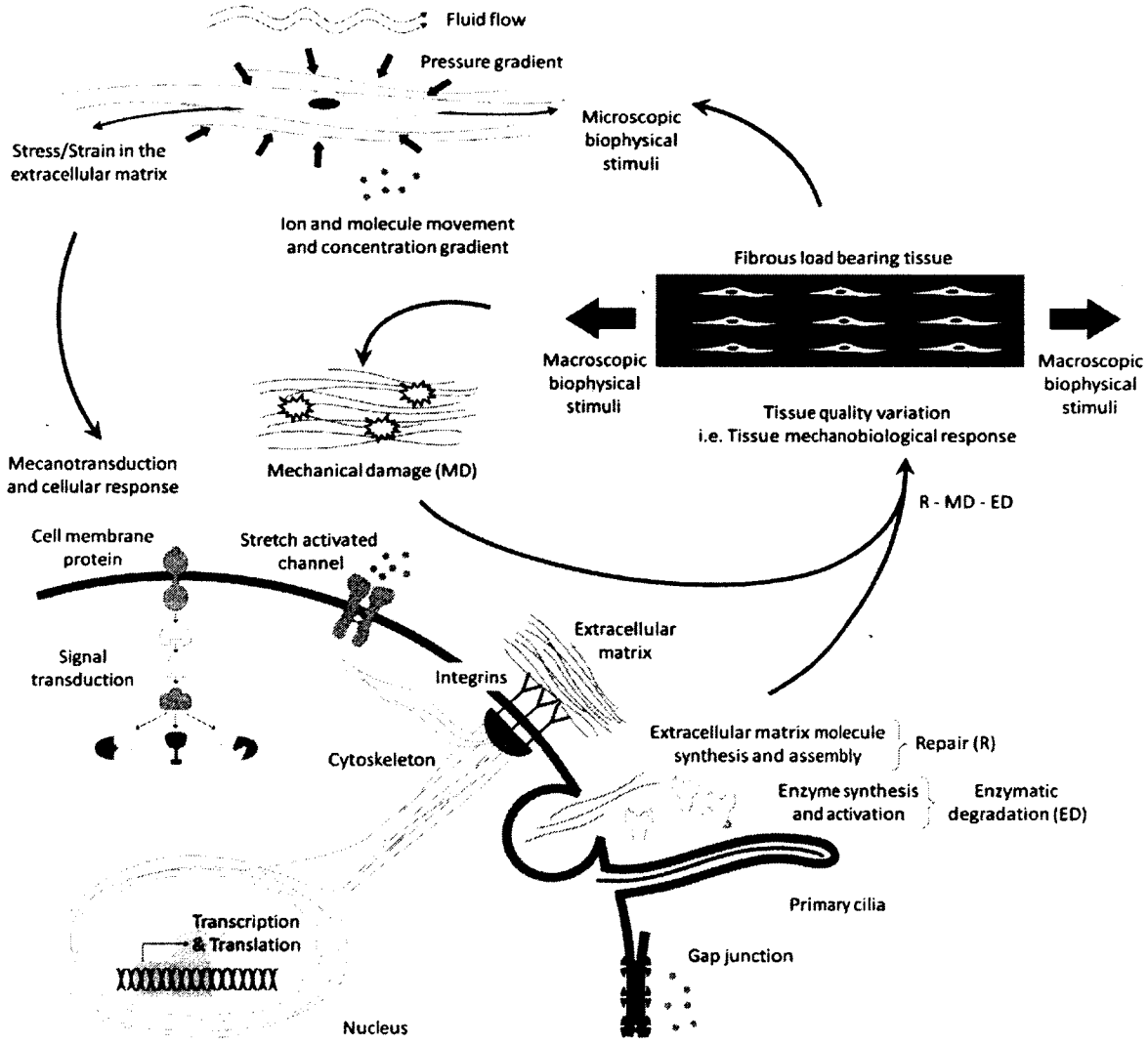

Figure 3-A-1: Illustration of the mechanobiological response of FLBT under biophysical. Under macroscopic biophysical stimuli, the inert extracellular matrix (ECM) undergoes mechanical degradation (MD). At the same time, the ECM reduces the macroscopic stimuli applied to the tissue as a whole into microscopic stimuli detected by the cells. This process is called mechanotransduction. The resulting biochemical signals instruct the cells to repair (R) or use enzymatic degradation (ED) on the ECM. As the tissue is altered in response to stimuli, the quality changes. Thus, the microscopic stimuli, biochemical signals, R, ED and MD also progress and so on.

Declaration of Interest

The authors have nothing to disclose.

4. Mechanical characterization tests of physiological amplitude conducted at regular intervals can affect tissue response to mechanobiological stimuli

4.1 Avant-propos

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Revue: Biomechanics and modeling in mechanobiology

Titre en francais: Des essais de caractérisation mécanique d'amplitude physiologique réalisés à intervalles réguliers peuvent influencer la réponse tissulaire aux stimuli mécanobiologiques

Résumé français:

La mécanobiologie joue un rôle majeur dans le domaine musculo-squelettique, notamment en génie tissulaire de même que dans la prévention et la guérison des blessures. Dans l'étude de la mécanobiologie des tissus en bioréacteur, la caractérisation de l'évolution des tissus est essentielle pour comprendre et éventuellement prédire leur réponse aux stimuli mécaniques, mais malheureusement, les méthodes utilisées sont souvent destructives (e.g. histologie ou essai de rupture). Ce serait néanmoins un grand avantage d'avoir un portait de l'évolution de la qualité des tissus dans le temps. Il y a cependant un paradoxe lors de la mesure de l'évolution des tissus vivants dans le temps : comment pouvons-nous mesurer précisément l'évolution des tissus dans le temps s'ils réagissent aussi à nos méthodes de mesure? Les méthodes utilisées pour observer l'évolution des tissues dans le temps peuvent induire un biais qui peut varier en fonction du protocole d'observation. Dans cette étude, nous avons

examiné l'hypothèse que des essais de relaxation de contrainte d'amplitude physiologique réalisés à des intervalles réguliers entre les périodes de stimulation ne modifient pas l'évolution des tissus dans le temps. Nous avons soumis des tendons sains et vivants à des stimuli mécaniques d'amplitudes physiologiques à l'intérieur d'un bioréacteur pendant 3 jours. Nous avons regroupé les tendons selon le protocole de caractérisation (0 ou 24 essais de relaxation d'amplitude physiologique chaque jour) et nous avons comparé l'évolution des groupes dans le temps. Les essais de relaxation de contraintes d'amplitude physiologique ont modifié l'évolution des tendons en réponse aux stimuli mécaniques *in vitro*. De façon générale, le module pointe a augmenté dans le temps pour le groupe de 0 essai de relaxation de contrainte alors qu'il a d'abord diminué puis légèrement augmenté pour le groupe de 24 essais de relaxation de contrainte chaque jour. En conclusion, l'insertion d'essais de relaxation de contrainte d'amplitude physiologique pendant les périodes de repos entre les stimuli mécaniques peut influencer l'évolution des tissus dans le temps.

Title: Mechanical characterization tests of physiological amplitude conducted at regular intervals can affect tissue response to mechanobiological stimuli

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4.2 Abstract

Mechanobiology plays a major role in skeletal tissue engineering; it is also an important field of study in the prevention and healing of certain musculoskeletal disorders. In bioreactor studies of tissue mechanobiology, characterization of tissue progression is essential to understanding and eventually predicting its response to mechanical stimuli but unfortunately, the methods used are often destructive (e.g. histology or rupture test). It would nevertheless be a great advantage to have a portrait of tissue quality progression over time. There is however a paradox in measuring progression in live tissue: how can we accurately measure tissue progression over time if the tissue is also reacting to our measurement methods? The methods used to observe tissue progression over time can introduce a bias that may even vary depending on the observation protocol. In this study, we investigated the hypothesis that stress relaxation tests at physiological amplitudes conducted at regular intervals between stimulation periods do not modify tissue progression over time. We subjected live healthy tendons to mechanical stimuli at physiological amplitudes inside a bioreactor for 3 days. We grouped the tendons based on the characterization protocol (0 or 24 stress relaxation tests at physiological amplitudes each day) and compared group progression over time. Stress relaxation tests at physiological amplitudes modified the tendon response to mechanical stimulation *in vitro*. Generally, peak modulus increased over time for 0 stress relaxation tests each day, whereas it first decreased and later lithely increased for 24 stress relaxation tests each day. Therefore, inserting stress relaxation tests at physiological amplitudes during rest periods between mechanical stimulation may influence tissue progression over time.

Key words

Tendon, Mechanobiology, Mechanical properties, Characterization, Progression, Stress relaxation

4.3 Introduction

Skeletal mechanobiology investigates how load-bearing tissues are produced, maintained and adapted by cellular activity in response to physical stimuli (van der Meulen and Huiskes 2002). It plays a major role in tissue engineering where it allows us to develop prosthetic organs that can carry out the functions of natural tissues in the body (Freed et al 2006; Guilak et al 2003). It is a significant field of study in the prevention and healing of certain musculoskeletal disorders, since many of these disorders are associated with inadequate mechanical loading, as reviewed by Wang and Thamppatty (Wang 2006; Wang and Thampatty 2006). In mechanobiology, characterization of tissue progression is essential to understanding and eventually predicting tissue response to mechanical stimuli. Various methods are used: optical and electron microscopy to characterize tissue structure at different scales; Western blot and hydroxyproline assay to characterize tissue composition; Northern blot and real-time PCR to characterize gene expression; traction tests to characterize mechanical properties (e.g. Young modulus and ultimate tensile stress and strain).

Unfortunately, these methods are often destructive (Kortsmit et al 2009). We therefore cannot use them at regular intervals on the same sample to characterize progression over time. Moreover, it is sometimes impossible to combine any two destructive characterization methods: this either limits the available information on the tissue quality or requires a greater number of specimens. For example, the traction test damages tissue structure and introduces bias to microscopic analysis. Another example is when we take a biopsy for microscopic analysis on a tissue sample intended for a traction test: this weakens the sample and skews the mechanical characterization.

A few authors have already recognized the need to describe tissue quality progression (Cousineau-Pelletier and Langelier 2010; Guilak et al 2003; Kortsmit et al 2009; Lujann et al 2011; Shulz et al 2008). It would certainly be useful to have a portrait of the progression of tissues over time, which would take the form of homeostasis (no change), degeneration or improvement. In the special case of load bearing tissues, the characterization of the mechanical aspects of tissue quality would inform us of the

tissue's capability to fulfill its primary function, i.e. to transmit, damp and/or support loads (Guilak et al 2003; Lujann et al 2011). To achieve this objective, non-destructive mechanical tests such as stress relaxation, creep or dynamic tests of physiological amplitudes can be used. When conducted at regular time intervals, these tests provide a picture of tissue mechanical properties over time.

In the field of cell mechanics, Bao and Suresh (2003) formulated a paradox: "how can we measure the mechanical behaviour of living cells if they react to our measurement tools?" A similar paradox can be formulated for the progression of live tissue: how can we measure tissue progression over time if it reacts to our measurement methods? At the moment, we do not know if the "observer effect" can be set aside, since we do not know if existing methods to observe tissue progression over time modify tissue progression in a negligible way or not. This unanswered question is important because the observer effect could introduce a bias between reality and observation. Moreover, this bias could vary with the observation protocol, and make it difficult to compare results between studies.

We investigated the hypothesis that stress relaxation tests at physiological amplitudes conducted at regular intervals do not modify tissue progression over time. This hypothesis is based on previous work done on freshly isolated articular cartilage where it was observed that small amplitude stress relaxation tests conducted repetitively over a 12-hour period superposed very closely as if they did not impact the next one (Langelier and Buschmann 2003).

To test our hypothesis, we subjected live tendons to mechanical stimulation at physiological amplitudes inside a bioreactor for 3 days. We divided the tendons into two groups. In each group, we included 0 or 24 stress relaxation tests at physiological amplitudes each day. We then compared group progression over time.

4.4 Materials and Methods

Tissue Explant Isolation and Preparation

All animal care and handling were approved by the Council of Animal Protection at the Université de Sherbrooke. Eight Sprague-Dawley rats between the ages of 4 and 6 months were sacrificed using carbon dioxide. Tendon isolation and preparation were conducted as described in previous studies (Bruneau et al 2010, Cousineau-Pelletier and Langelier 2010). All manipulations presented in this section were performed in cold D-PBS 1X (311-410-CL; Wisent Inc., St-Bruno, Canada) containing 1g/L glucose (609-037-EL; Wisent Inc.) and 1% antibiotic-antimycotic (15240-062; Invitrogen, Burlington, Canada). Four tendons were isolated from each rat tail within an hour of resection (Figure 4-1). Following isolation, the cross-sectional tendon areas were evaluated using an optical micrometer (Parent et al 2010). They were then washed 5 times under the biosafety cabinet. For mechanical characterization and stimulation, the tendons were transferred into the bioreactor (Parent et al 2011). The ends of the tendons were wound around cylinder-shaped anchors and allowed to dry briefly on the top face of the anchors. A small drop of ethyl cyanoacrylate (10300; Krazy Glue, Columbus, OH) was applied to the portion of the tendon at the top of the anchor.

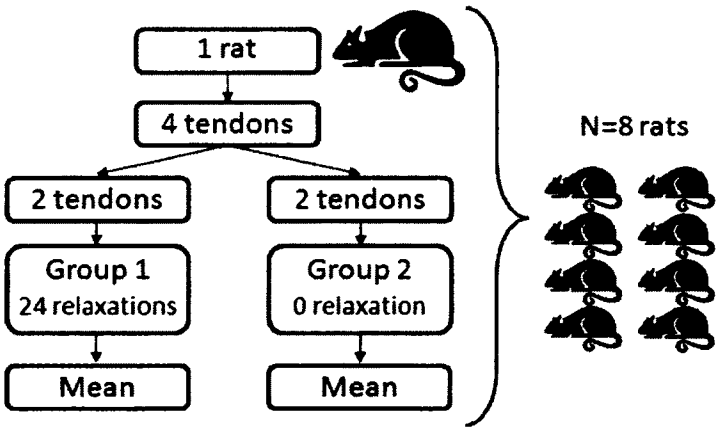

Figure 4-1: Number and distribution of the tendons for each rat. For statistical analysis of the peak-to-peak modulus between two groups, we used Wilcoxon matched-pairs signed rank test.

Tissue Culture

During the experiment, the tendons were maintained in DMEM (12800-017; Invitrogen, Burlington, Canada) supplemented with 3.7 g/L of sodium bicarbonate (600-105-CG; Wisent Inc.), 10% FBS (090150; Wisent Inc.), and 1% antibiotic-antimycotic solution.

Mechanical Stimulation

After temperature stabilization in the bioreactor at 37°C, the specimens were subjected to the following stimulation protocol. The initial zero strain reference was defined by achieving a tension load of 3g at equilibrium. Preconditioning was performed with a series of 120 sinusoidal waves at two different amplitudes (60 cycles at 1% strain; 60 cycles at 2% strain) executed at 1 Hz. The final zero strain reference was defined by again reaching a tension load of 3g at equilibrium. Thereafter, the 3-day stimulation protocol was used: each day, the tendons were subjected to four periods of 6h each composed of 30min of stimulation (sine wave pattern; 1.2% strain; 1Hz) and 5 h 30 min of rest (0% strain).

Impact of Stress Relaxation Tests and Recovery Periods

To highlight the impact of mechanical tests at physiological amplitudes on the mechanobiological response, we included stress relaxation tests (1%/s strain rate, 1.2% strain, 30s pause) in the stimulation protocol. The strain amplitude was selected based on preliminary traction tests (data not shown) in which the linear portion of the stress–strain curve spread up to about 1.5% strain. We divided the tendons into two groups (Figure 4-1). In group 1 (N= 8), 24 stress relaxation tests were integrated each day (1 per hour). In group 2 (N= 8), no relaxation tests were integrated (Figure 4-2).

Mechanical Characterization of Tissue Progression

To characterize the changes in mechanical properties over the 3-day period, we adapted the method developed by Cousineau-Pelletier and Langelier (2010). We evaluated the changes in the peak-to-peak modulus produced by the stimulation protocol. We used the modulus in the last ten cycles of the first stimulation period as a

reference (M_{ref}). Every 6 hours, we compared the modulus for the last ten cycles of stimulation (M) to this reference value (Figure 4-3).

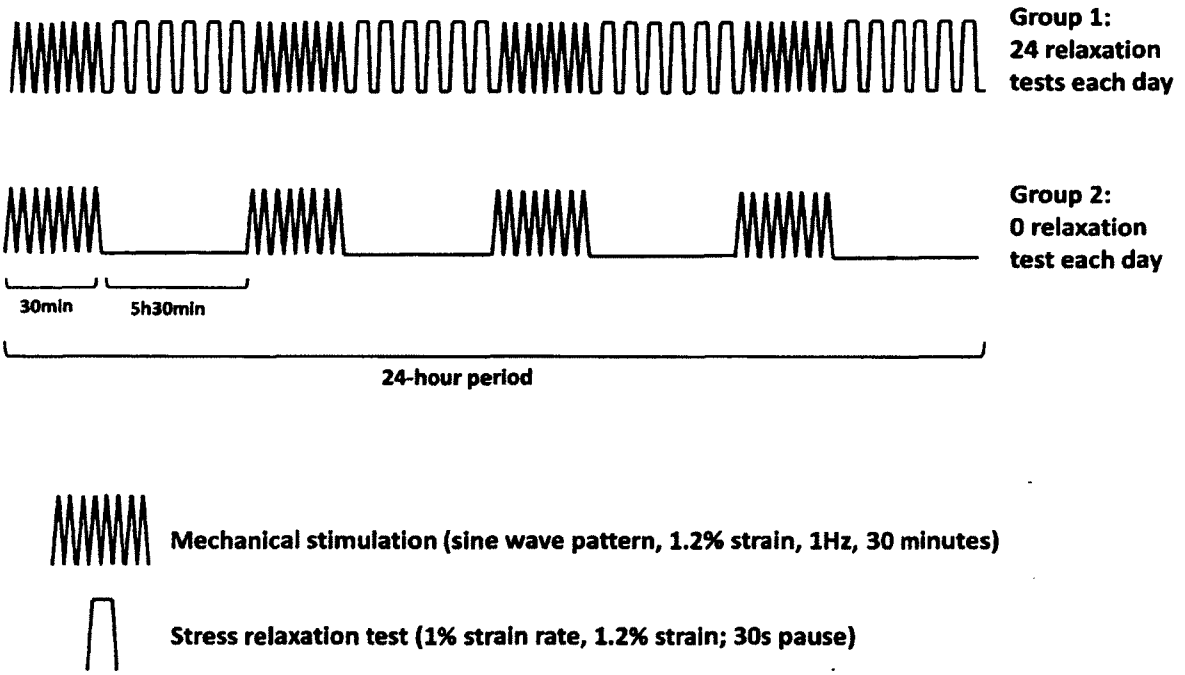

Figure 4-2: Integration of stress relaxation tests between stimulations

We calculated the peak-to-peak modulus as peak-to-peak stress divided by peak-to-peak strain. The stresses were evaluated as the ratio of the forces measured with load cells over the initial tissue cross-sectional areas. The strains were calculated as the ratio of the changes in length measured with encoders over the initial tendon length. We calculated the response of each tendon as:

$$\text{Change}\ (\%) = \frac{M}{M_{ref}} \times 100 \qquad (1)$$

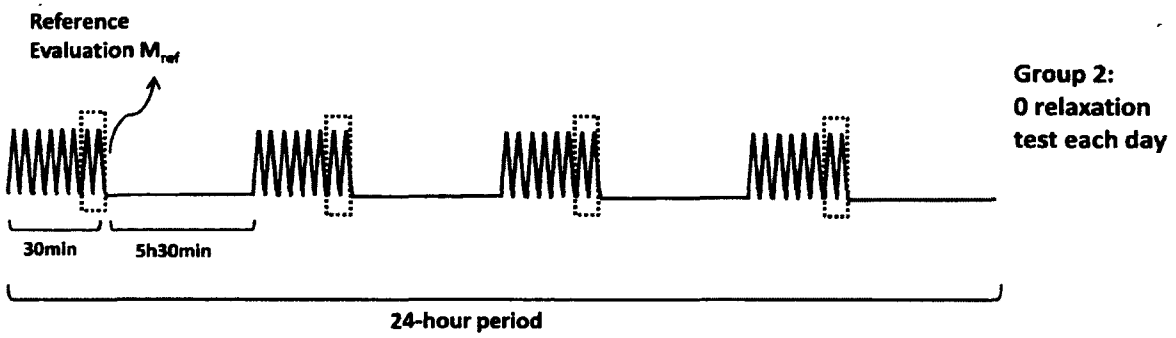

Figure 4-3: Evaluation of changes in peak modulus. The mean peak-to-peak modulus in the last 10 cycles of the first stimulation period was used as a reference. Every 6 hours, the mean peak-to-peak modulus in the last 10 cycles of stimulation was compared to the reference value

Statistical Analysis

We used the Wilcoxon matched pair signed rank test to verify whether there is a significant difference between two tendon groups. We used the non-parametric test since the population was small and did not have a normal distribution. For each rat, the data sets obtained for two tendons were averaged in each group (Figure 4-1). Thus, for each rat, a pair of data sets was always available: one set for 0-relaxation group, and another one for 24-relaxation group. The significance was set at $p < 0.05$.

4.5 Results

Change in Peak Modulus

The number of stress relaxation tests influences changes in the peak-to-peak modulus as shown in Figure 4-4. Tendons subjected to 0 stress relaxation tests each day saw their peak modulus increase to approximately 115% in three days. However, over the same period, tendons subjected to 24 stress relaxation tests each day saw their peak modulus decrease to approximately 93.5%. The difference between the peak modulus of two groups was significant at all time points.

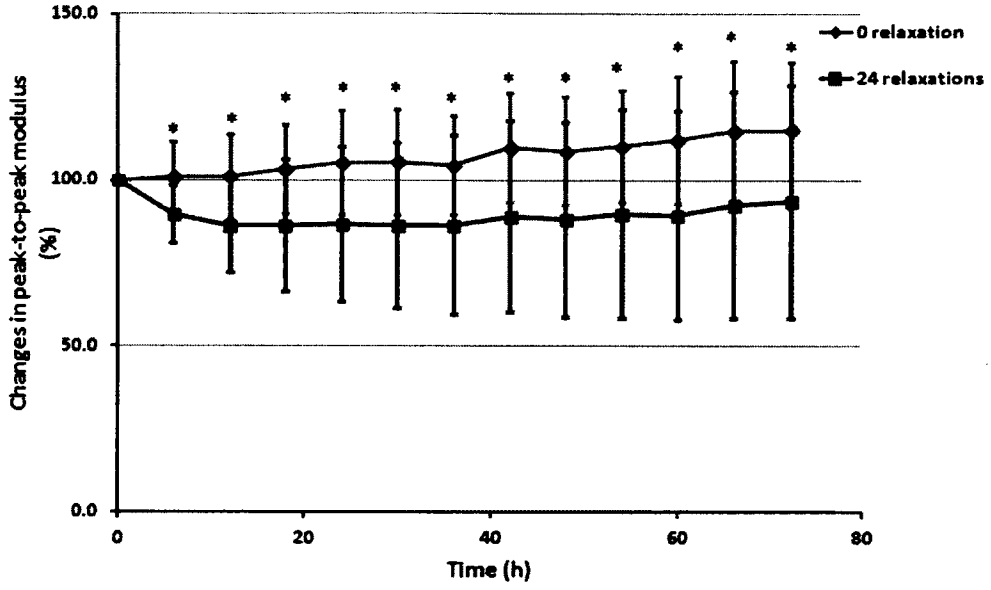

Figure 4-4: Changes in peak modulus of each group (mean ± SD). At the end of day 3, changes in peak modulus were 93.5 ±35.1% for group 1, and 115 ± 20.5% for group 2. Stars indicate significant differences between the 2 groups.

4.6 Discussion

This study shows that stress relaxation tests at physiological amplitudes can modify the response of healthy tendons to *in vitro* mechanical stimulation. The changes in measured peak-to-peak modulus varied with the number of repetitions per day as a negative impact on stress recovery could be measured with the 24 daily stress relaxation tests.

It is worth mentioning that our modification to the method of characterizing the changes in mechanical properties over the 3-day period did not alter the conclusion of the study. Previously (Cousineau-Pelletier and Langelier 2011), we had compared the mean peak-to-peak stress values of the last 5 minutes of stimulation with the reference value, which was the maximum peak-to-peak stress value of the first minute of the whole stimulation. We implemented the "reference" modification to eliminate the effect of dynamic relaxation on the reference value and to minimize the intra-animal variation which is smaller after dynamic relaxation. Also, we implemented the "modulus vs stress

modification" to account for strain. We statistically tested the results calculated in this manner. Results for both groups (0 vs 24 relaxations/day) were significantly different at 18, 30, 42, 54 and 60h with $p<0.05$; and at 12, 24, 36, 48 and 66h with $p<0.08$. The difference in the statistical significance of these results compared to our new method of evaluating changes in mechanical properties can be explained by the pairing, which was significant with the new method ($p<0.05$) but not with the older method. Therefore, our conclusion remains unchanged: stress relaxation tests at physiological amplitudes can modify the response of healthy tendons to in vitro mechanical stimulation.

These results may have implications in other fields such as the study of time - dependent spine stability and related risks of injury as well as lower back pain. As reviewed by Solomonow (2011), there are two categories of spine stabilizers. The first category includes the passive components: the ligaments, disks, capsules and fascia, which stabilize the spine through their viscoelastic properties. The second category includes the dynamic components: muscles and their sensory-motor control, which stabilize the spine through co-contraction, muscular stiffness, intra-abdominal pressure, and compressive force on disks. The tissues forming the passive components are exposed to stretching during daily activities, inducing spine laxity. Stretching affects the sensitivity of mechanoreceptors as well as the control of muscular activity, and thus may influence spine stability. Rest periods are essential for both passive and active component recovery, but should the recovery period be complete rest or low amplitude activities? In seeking to answer this question, our study reveals that the number of repetitions of low amplitude activities may have a substantial impact on recovery time and should therefore not be neglected.

The results presented in this study can be explained by two mechanisms: viscoelasticity and cellular activity. When submitted to dynamic stimulations under strain control, tendons experience stress relaxation. This phenomenon, explained by the viscoelastic nature of tendons, can be illustrated as a string which elongates slightly at each cycle (up to a maximum corresponding to the stimulation amplitude). Consequently, the measured stress decreases at each cycle because the string is under decreasing tension. At rest, between the dynamic stimulation periods, the string shortens and recovers

from stress. This recovery is partly attributed to viscoelasticity and partly to cellular activity. In a previous study we showed that stress recovery is greatly reduced in the absence of cellular activity (Cousineau-Pelletier and Langelier 2010).

The inclusion of stress relaxation tests can perturb stress recovery even though the physiological amplitude is slight (1.2%) and the period is short (30s) in comparison to the rest period (5h30min). Stress relaxation tests may impede stress recovery by slightly lengthening the tendon at each occurrence. This in turn may impact cellular activity through stress decrease/deprivation at the cellular scale, which has been shown to upregulate collagenase mRNA expression and protein synthesis (Lavagnino et al 2003, 2005a; Arnoczky et al 2004; Lavagnino and Arnoczky 2005). As a consequence, the extracellular matrix, cell-matrix interactions and mechanotransduction may be degraded (Arnoczky et al 2007) and a vicious cycle may ensue.

Of course, many questions are raised and remain unanswered regarding the impact of stress relaxation tests on tissue mechanobiological response. For example, although we suspect that increasing strain rate, amplitude and length of the test and decreasing rest periods between tests increases the impact on tissue response, we do not know the precise effects of these parameters. We also do not know the effect of the initial tissue quality (sedentary or a high level of fitness, young or old, healthy or diabetics, etc). The same questions apply to creep tests and dynamic tests.

Until more is known on the subject, the stimulations themselves should be used for mechanical characterization as in previous studies (Androjna et al 2007; Cousineau-Pelletier et al 2010; Devkota et al 2007; McCulloch et al 2004; Preiss-Bloom et al 2009; Shulz et al 2008; Tran et al 2011) and in the present study to analyze peak-to-peak modulus (Figure 4-4). Since stress relaxation tests introduce new mechanical energy to the tissues, they must be considered as stimulations and their impact must not be neglected. Therefore, researchers using them for mechanical characterization need to describe their characterization protocol precisely and verify its impact on the results and conclusion of their study. Obviously, the solution of using the stimulations for characterization is not a perfect one. Due to the nonlinear properties of tissues, a

comparison between different stimulation conditions may be complex. However, this observation process has an indisputable advantage: it does not interfere with tissue progression.

Tissue characterization during bioreactor confinement is emergent and has not yet been standardized. However, we do encourage researchers to implement this means of gathering more information on tissue progression over time while using the stimulations for characterization.

4.7 Conclusion

This study has shown that inserting stress relaxation tests at physiological amplitudes during rest periods between mechanical stimulations may influence tissue progression over time. As of today, to the paradoxical question *how can we measure the tissue progression over time if it responds to our measurement methods?,* the answer would be to use the mechanical stimuli themselves as part of the study design. In this way, more information can be gathered on tissue progression without introducing new energy to the tissue.

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5. Unpublished microscopy results

In this chapter, we present the data which are not included in the second article. These data include the methods we used at Biometiss to characterize tissue quality quantitatively and semi-quantitatively based on microscopic images. With these characterization methods we tried to evaluate if there were differences between the two groups of stimulated tendons: 0-relaxation group, and 24-relaxations group.

The tissue quality analyses were conducted on OM and TEM micrographs. We used standard tissue preparation methods (Cousineau-Pelletier and Langelier 2009). Briefly, the samples were simply fixed by being soaked in formalin (OM), or glutaraldehyde (TEM). Then the samples were rinsed inside a buffer solution. The next step was dehydration followed by embedding the samples in a support medium for thin sectioning. The support medium was paraffin (OM) or epoxy resin (TEM). Finally, samples were cut and stained with Hematoxylin/Eosin (H & E) (OM) or uranyl acetate and lead citrate (TEM) to add contrast. The microscopic images of samples were used for quantitative and semi-quantitative characterizations.

Quantitative and semi-quantitative characterization of tissue structural properties could be very useful to avoid inconsistency in diagnosis between specialists resulting from qualitative (descriptive) characterization.

We divided this chapter in two sections. In the first section, we present quantitative methods to evaluate tissue structural quality using the "National Instrument vision assistant" (NI-Vision) program. In the second section, we present the semi-quantitative method, i.e. the modified Bonar-Movin scoring method to evaluate tissue structural and cellular quality.

5.1 NI vision for tissue structural quality

Using NI-Vision software, we tried to calculate collagen fibril density on OM (longitudinal view) and TEM (cross-sectional view) images of tendons.

For each image we chose a region of interest (ROI). It should be noted that areas with damages caused by preparation were not included (e.g. damage caused by the knife; a fold in the sample). In selected ROIs, fibrils and spaces between them were found by contrast and separated into two categories: fibril (black); and background (red) (Figure 5-1). Fibril density was calculated by dividing number of collagen fibril pixels (black) by number of pixels in the whole area (black+red).

However, we encountered a challenge to set an appropriate and repetitive contrast and, therefore, the proper fitting of the black-red image with the origin image. Figure 5-1 is an example of this challenge for OM images. By choosing identical ROIs in both images, but with different contrasts, the resulting fiber densities were highly different: 78% vs. 97%. This large variation could lead to inadequacy in quantitatively characterizing ECM structure. Therefore, we found this method inappropriate for our purpose.

We encountered the same problem with TEM images. We therefore tried another method used in histological analysis of tendinopathy: the semi-quantitative method of Bonar-Movin scale.

5.2 Bonar-movin for structural and cellular quality

5.2.1 Using standard OM and TEM methods

Histological, semi-quantitative analyses were performed on microscopic images (OM and TEM) of stimulated tendons. For this analysis we modified Bonar-Movin scoring scale. The variables we used in our scoring systems for OM images were: 1) cell morphology, 2) cell aggregation, 3) cell density, 4) fiber waviness, and 5) between-fiber spaces. The magnification used for the variable scoring was set at 20x, except for "cell morphology". To have a more realistic scoring for cell morphology we used a higher magnification of 40x. Each variable was scored between 0 and 3, as 0 corresponds the normal feature of the tendons and 3 corresponds to the most severe damage could be detected in our samples (Table 5-1)

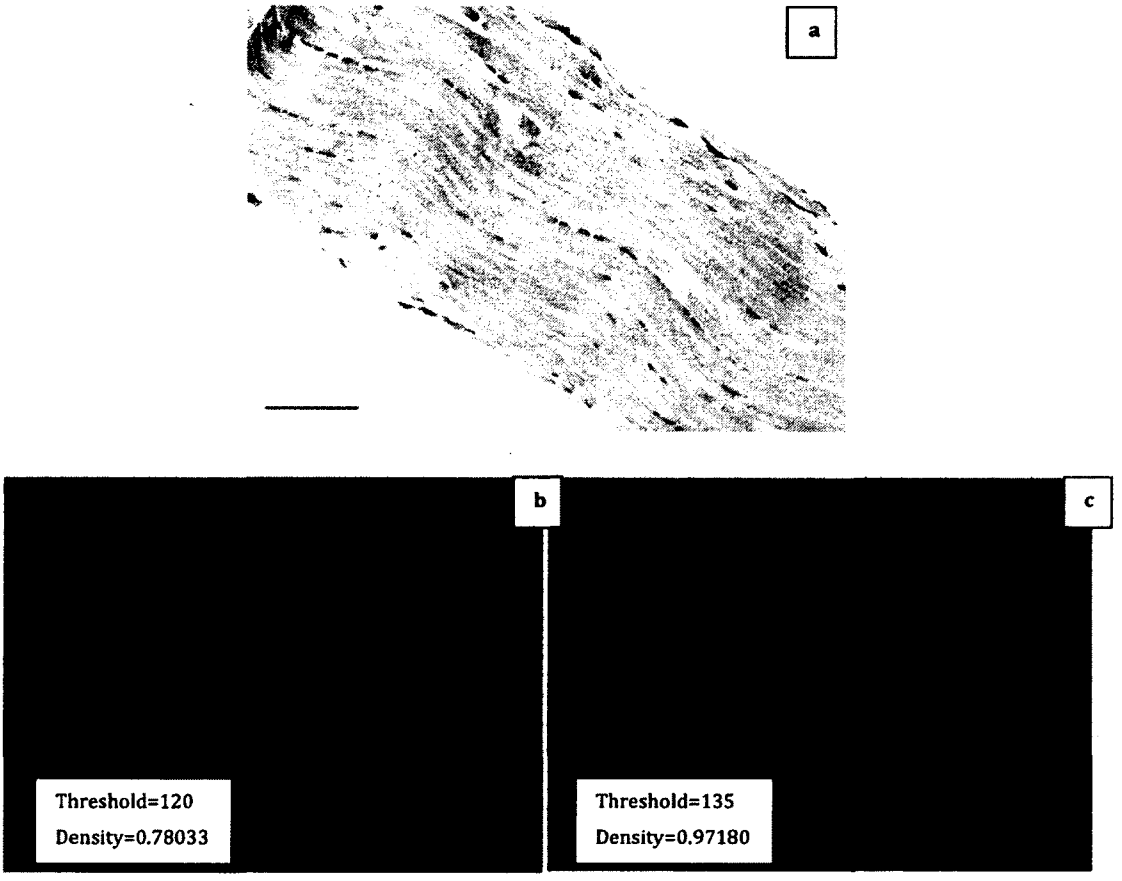

Figure 5-1: Impact of contrast on density results. a. Longitudinal section of H&E stained tendon under light microscopy. b, c. black-red images with different contrasts of original image (a). Selected ROIs in images b and c are identical, but with different contrasts. The resulting fiber densities are highly different: 78% vs. 97%. Bar = 200 μm.

Table 5-1: Modified Bonar-Movin scoring scale in this research

	OM images (longitudinal sections)					**TEM images (Cross-sectionnal sections)**
	Cell morphology	**Cell aggregation**	**Cell density**	**Fiber waviness**	**Between-fiber spaces**	**Fiber density**
0	Elongated	Long lines of cells	Low density	Straight	Low space	High density
3	Round	Isolated cells (OR small lines of cells)	High density	Wavy	Large space	Low density

Two authors scored the images. After two weeks, they scored the same images again. If there was a difference of more than one degree between the 4 readings of a variable, it was scored for the final time with consultation of both authors. Figure 5-2 and Figure 5-3 are examples of our scoring scale taken from one reading from one author (out of four readings).

24 relaxations

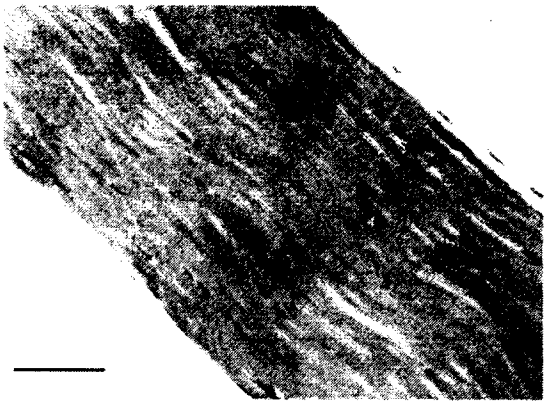

Figure 5-2: light micrograph of rat tail tendon from 0-relaxation group. Cell morphology:1; Cell aggregation:2; Cell density:1; Fiber waviness:3; Space between fiber:1. Bar = 200 μm

0 relaxation

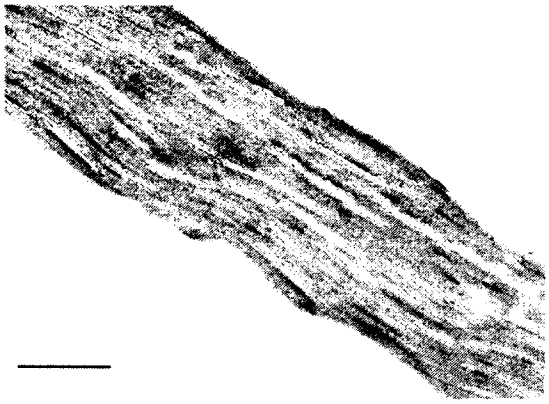

Figure 5-3: light micrograph of rat tail tendon from 24-relaxation group. Cell morphology:1; Cell aggregation:1; Cell density:1; Fiber waviness:1; Space between fiber:2. Bar = 200 μm

The global results obtained using this method is presented in figures Figure 5-4 toFigure 5-9.

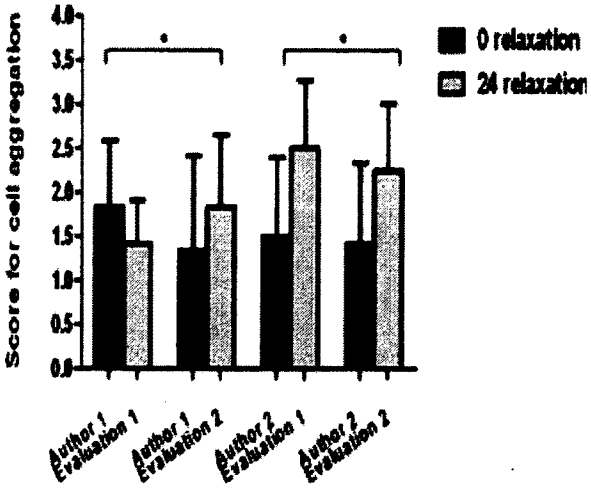

Figure 5-4: Modified Bonar-Movin scores for *cell aggregation* on OM images. * shows the agreement of two evaluations by the same author. ** shows the agreement of all four evaluations.

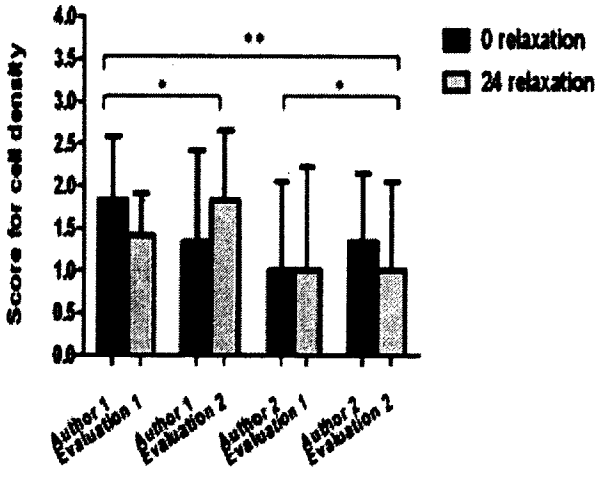

Figure 5-5: Modified Bonar-Movin scores for *cell density* on OM images. * shows the agreement of two evaluations by the same author. ** shows the agreement of all four evaluations.

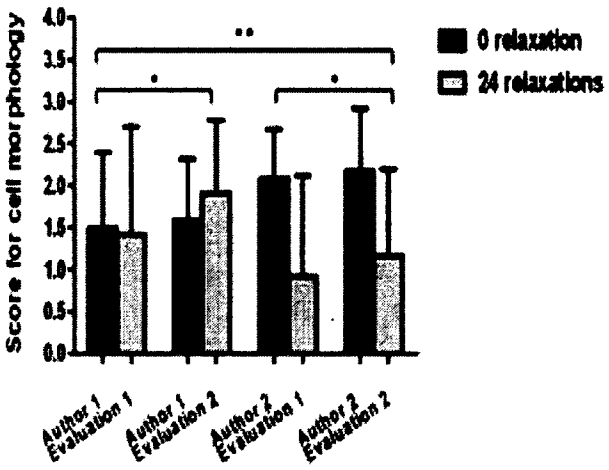

Figure 5-6: Modified Bonar-Movin scores for *cell morphology* on OM images. * shows the agreement of two evaluations by the same author. ** shows the agreement of all four evaluations.

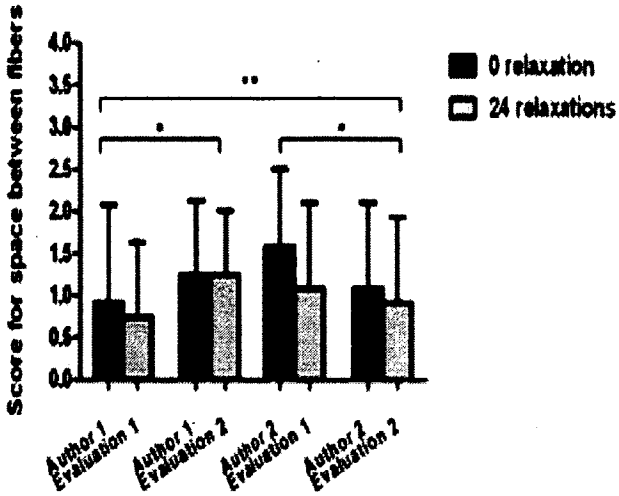

Figure 5-7: Modified Bonar-Movin scores for *space between fibers* on OM images. * shows the agreement of two evaluations by the same author. ** shows the agreement of all four evaluations.

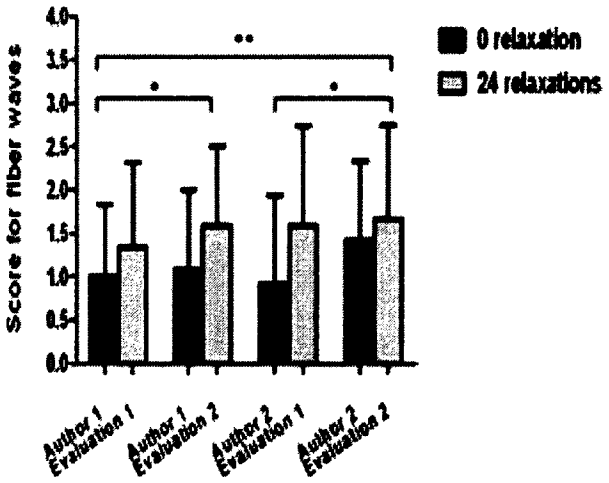

Figure 5-8: Modified Bonar-Movin scores for *fiber waves* on OM images. * shows the agreement of two evaluations by the same author. ** shows the agreement of all four evaluations.

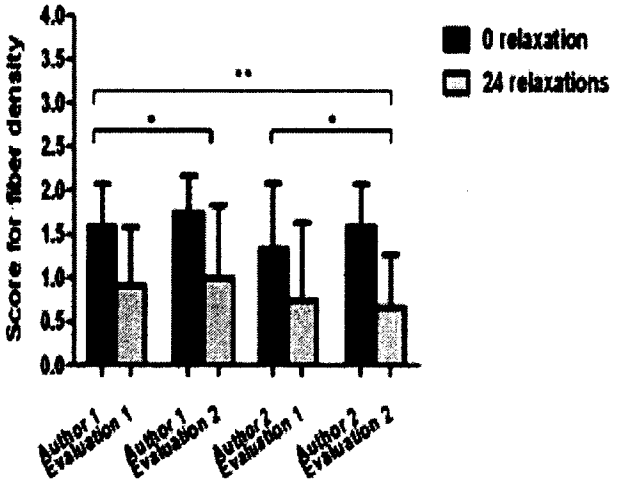

Figure 5-9: Modified Bonar-Movin scores for *fiber density* on TEM images. * shows the agreement of two evaluations by the same author. ** shows the agreement of all four evaluations.

The agreement of four evaluations was assessed by the Intra-class correlation (ICC) test. There was a perfect agreement between the four evaluations for all variables, except for cell aggregation (c.f. Figure 5-4 and Table 5-2). For cell aggregation there was a perfect agreement between the two evaluations of each author but there was no agreement between the four evaluations i.e. there was no agreement between authors in scoring cell aggregation.

Table 5-2: ICC scores for each variable (1 indicates perfect agreement and 0 indicates no agreement. For this study the ICC was set at 0.80)

<table>
<tr><th></th><th colspan="8">OM images (longitudinal sections)</th><th colspan="4">TEM images (Cross-sectionnal sections)</th></tr>
<tr><th></th><th colspan="2">Cell morphology</th><th colspan="2">Cell aggregation</th><th colspan="2">Cell density</th><th colspan="2">Fiber waviness</th><th colspan="2">Between-fiber spaces</th><th colspan="2">Fiber density</th></tr>
<tr><td>Author 1, evaluation 1</td><td rowspan="2">0.81</td><td rowspan="4">0.81</td><td rowspan="2">0.81</td><td rowspan="4">0.17</td><td rowspan="2">0.81</td><td rowspan="4">0.84</td><td rowspan="2">0.95</td><td rowspan="4">0.96</td><td rowspan="2">0.88</td><td rowspan="4">0.94</td><td rowspan="2">0.96</td><td rowspan="4">0.93</td></tr>
<tr><td>Author 1, evaluation 2</td></tr>
<tr><td>Author 2, evaluation 1</td><td rowspan="2">0.87</td><td rowspan="2">0.89</td><td rowspan="2">0.93</td><td rowspan="2">0.91</td><td rowspan="2">0.85</td><td rowspan="2">0.91</td></tr>
<tr><td>Author 2, evaluation 2</td></tr>
</table>

The significance of the difference between the scores of two groups were assesses by the Wilcoxon matched pair sign rank test. We conducted the test on each of the four scorings, the mean of two scorings from each author, and the average of all four readings from both authors. There was no significant difference between tissue qualities (including both ECM and cell qualities) of 0 and 24 relaxation groups (p-value was set at 0.05).

We chose not to publish these data because of some concerns about their reliability. For example, although four readings statistically agreed for most of the variables, i.e. the

four scorings were statistically repetitive, the trend of difference between 0 and 24 relaxation groups was not always repetitive between 4 evaluations (Figures 5-4 to 5-6).

One possible explanation is that the duration of mechanical stimulation, i.e. 3 days, was not long enough to change tissue structural properties. Thus, the tissues were not very damaged and, moreover, our scoring scale was based on our most damaged tissues. So, the score of 3 was given for not so extensively damaged tissue. Therefore, it was difficult to classify the specimens because they all had similar structure.

Another possibility is that rat tail tendon preparation for OM is very difficult. Since the samples are very small and hard, damage can occur during preparation. Figure 5-10 demonstrates a fresh tendon which was damaged during preparation. To overcome this challenge, other preparation methods (e.g. Fung's method (Fung, Wang et al. 2009)) could be used.

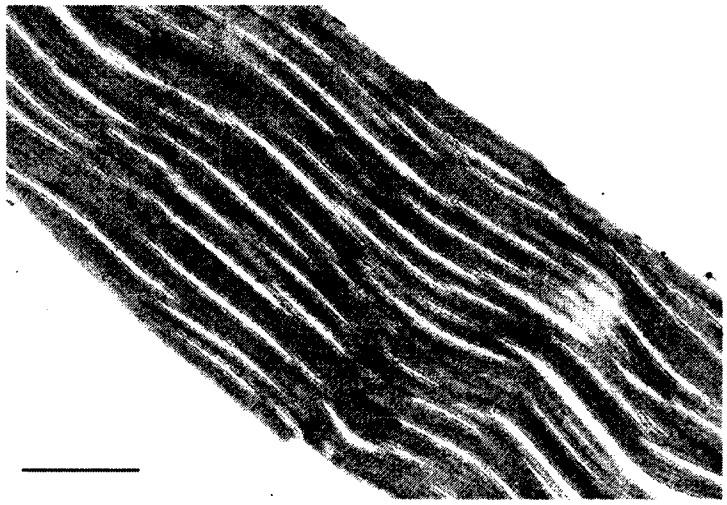

Figure 5-10: A fresh sample which was damaged during preparation. Bar = 200 μm

We also scored TEM images for their fiber density. Moreover, we studied cell morphology of TEM images (Figure 5-11). We did not publish these results either since these images were taken from very small sections of tendon. Therefore, these images may not represent the overall sample. To have more reliable results, many images should be taken from different spots on the specimen which would be very expensive

and time consuming. Moreover, the tissue preparation was not always adequate for cell observation and the responsible technician retired during this process.

Overally, semi-quantitative analysis was also inappropriate for our purpose

To overcome the challenges for studying cellular quality, we adapted an existing method to be able to observe whole tendon section. The method is fluorescent microscopy imaging of cryostat sectioned samples. It will be discussed in the following section.

Figure 5-11 : Electron micrograph of rat tail tendon cross-section. 3000 x magnification was used.

5.2.2 A new alternative method for cellular quality

To study cell morphology we adapted a technique using cryostat sectioning of DiI stained tissues (Vybrant CM-DiI cell-labeling solution, v22888) (C. M. McNEILLY 1996). DiI is a fluorescent dye for cell membrane labeling. The sections were cut in cross-sectional direction.

Here is the procedure we use at Biometiss for staining and cryostat sectioning of the tendons. After 3-day experiment, small biopsies (about 5mm-length) were taken from the tendons. Then they were fixed in formalin (10%) for 24 hours. Biopsies fixation was done first to preserve the cell shape. Fixed samples were then transferred to DiI

solution (1.5 micro liter DiI/1ml PBS). It should be noted that DiI can be applied on live and fixed tissues. Biopsies in DiI were incubated at 37°C for 24 hours. After 24 hours of incubation, the biopsies were ready for cryostat sectioning.

Cryostat is a device which is used for cutting very thin sections of frozen tissue. Another student at Biometiss already tried to cut cross-sections in paraffin but it did not work well. Thus, we tried cryostat technique for this purpose. To study cells of stained tissues we need to prepare slides of tissue in micron thickness. Tissues are therefore frozen inside cryostat chamber using tissue freezing medium. Frozen tissue is needed, because tissues should be hard enough to not get crushed during sectioning. The steps for cryostat sectioning are:

1) Cryostat device is turned on and the temperature is set at -30°C at least 45 min before starting the procedure. (it takes almost 45 min to reach -30°C);
2) The specimen is mounted on metal surface using OCT tissue freezing medium, and frozen inside the cryostat cooling chamber. The tissue is kept straight using tweezers inside the cooling chamber until it is frozen. Fixed samples, in addition to preserve the cell shape, are useful because they are easier to be kept straight.
3) Once frozen, the metal surface is mounted on the microtome.
4) The sample is sectioned into 50microns-thick. (we chose this thickness based on our preliminary experiments)
5) The sections are mounted on the microscopy slides using mounting media. "Vectashield hard set" (H-1500, Vector Laboratories Inc, Burlingame) mounting media is used at Biometiss. This mounting media has the ability to stain the nucleus with DAPI.
6) Microscopic images are captured from tendon cross-sections mounted in microscopy slides.

To observe prepared sectioned samples, we tried fluorescence microscopy in light and confocal microscopes. Figure 5-12 demonstrates a fluorescence image taken from light microscopy.

Figure 5-12 : Fluorescence micrograph of rat tail tendon section under light microscopy. The sample is stained with DiI.

Although the cells can be observed in this image, the exact shape of membrane is not clear because the focus cannot be done on the whole specimen thickness. Therefore, we tried another microscope, i.e. the confocal microscope, to observe our samples.

In confocal microscopy, successive images from different depths of the specimen can be taken. Therefore, each image is a very thin section, called as 0-thickness section, of the specimen cross-section. Imaging thin sections enable us to set focus for each image and thus observe cell morphology much easier on clear images. For example, the shape of the membrane and the processes of the cell could be recognized (Figure 5-13). Moreover, since the membrane edges are not blurry, it is possible to estimate the cell size and consequently compare different tendons. Finally, putting together the images from different depths, we get a 3D image of cells.

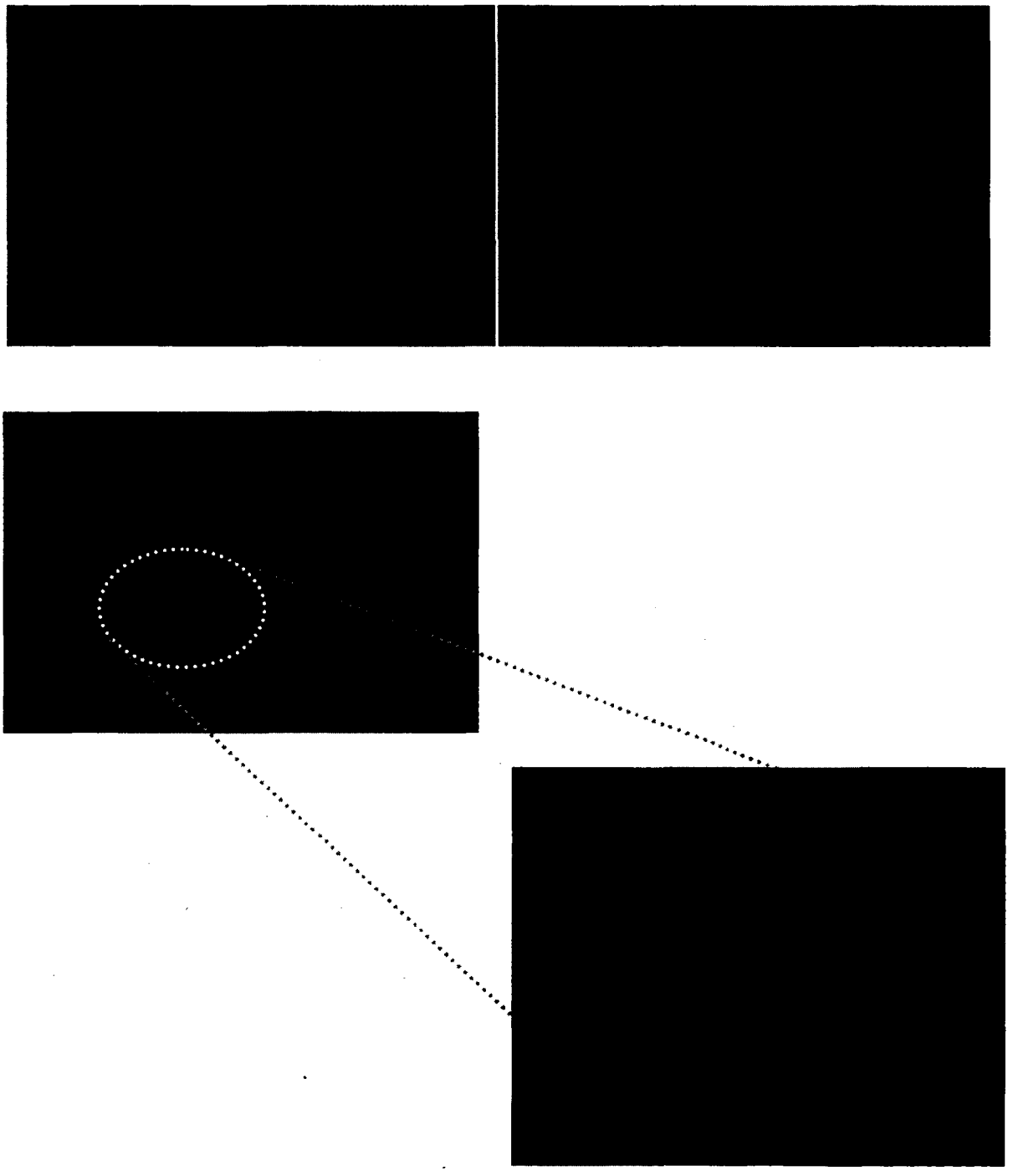

Figure 5-13: Fluorescence micrograph of rat tail tendon section under confocal microscopy. The picture is taken from very thin section of the tendon, referred as 0-thickness, at 10 micrometer depth. The sample is stained with DiI and DAPI. In (a) solely the nuclei of the cells are shown in blue. In (b) only membranes of the cells are shown in red. In (c) both membrane and nuclei of the cells are demonstrated.

However, we did not publish these data since we recently adapted this method at Biometiss and it is in preliminary steps and there are some challenges which still need to be resolved.

The first challenge is to cut tendon sections perpendicular to tendon longitudinal direction. It raises either from the difficulty of freezing tendon exactly perpendicular to the metal surface or from the difficulty of adjusting the cutting blade parallel to the sample section.

The second is that, although DiI has the ability to stain even fixed samples, it seems that DiI could not go through tendon sheath easily since the coloration at the center of the tendon is always less than around it. However, there is a concern that if we first stain and then fix the samples, the cell shape could undergo some changes since the staining process takes 24 hours.

Finally, there is an offset between discrete levels of tendon cross section images, i.e. the axis which connects the centroids of images is inclined. One probable possibility is that the samples are moving slightly inside microscopy slides. Also, it is possible that cut sections are not parallel to original tendon section. Since the desk of confocal microscopy is anti-shaking, the possibility of shaking desk has not been considered.

Hopefully, by removing these limitations, we will be able to expand our knowledge of cell study based on this method in future experiments.

6. Conclusion

In this chapter, concluding remarks based on the findings of this research project will be presented. In order to stress the results, next section will briefly review achievements of the research along with conclusions which were drawn from the results. Then, the novelty and contributions corresponding to these results are outlined. Afterwards, the burdens which limited this research project are explained. In the last section, some suggestions and propositions has been made in order to improve and follow up this research project.

Dans ce chapitre, sont présentées des conclusions basées sur les résultats de ce projet de recherche. Afin de mettre en lumière les résultats, la prochaine section résume brièvement les accomplissements de l'étude ainsi que les conclusions s'y rattachant. Puis, la nouveauté et les contributions associées à ces résultats sont exposées. Par la suite, les limitations de ce projet de recherche sont expliquées. Dans la dernière section, quelques suggestions et propositions sont faites pour améliorer et continuer ce projet de recherche.

6.1 Summary

In this section, a summary of the work which has been done in this project to achieve the objectives is presented.

The two objectives of this research study were:

1) To review the literature about viscoelasticity and viscoplasticity, and the way these two tissue properties affect live tissue response to mechanobiological stimulation;
2) To investigate whether diagnostic test of physiological amplitude affects live tissue response to mechanical stimulation.

A review article has been written to accomplish objective 1. In that article we mentioned that tissue exposes two macro-mechanical behaviors: viscoelastic and viscoplastic behavior. Tissue viscosity comes from frictional losses related to water content of ECM and/or to the relative motion of collagens. Tissue elasticity originates from collagen recoverable extension and collagen unit sliding past another. Tissue plasticity is a result of non-recoverable collagen extension related to high magnitude loading, or high repetitive loading which leads to ECM micro damages or non-reversible collagens sliding. Viscosity combines with either elasticity or plasticity in live tissue depending on tissue quality (combination of structural, compositional, and mechanical properties of tissue) and applied load.

Tissue viscoelasticity and viscoplasticity affect live tissue response to applied load. This response includes ECM response and cellular response. Briefly, the applied load on ECM is scaled down to be sensed by cells through mechanotransduction mechanisms. Even under condition of constant applied load, cell sense of applied load could change because of viscoelasticity and viscoplasticity. Stress-relaxation and creep are examples of this situation. Therefore, cell response, which is repair or degradation, changes and affects ECM structure. Consequently macro-mechanical behavior of tissue, i.e. viscoelasticity and viscoplasticity, are affected.

Therefore, we concluded that it is essential to take into account viscoelasticity and viscoplasticity of tissue while developing a tissue stimulation protocol for *in vitro* research and *in vivo* clinical applications. In other words, tissue progression could be affected by some parameters of the stimulation protocol because of these two behaviors. The parameters which should be highlighted in designing stimulation protocols are:

1) Control-type of stimulation: stress-controlled experiments affect tissue progression in a different manner than strain-controlled experiments.
2) Stimulus history: resting periods affect tissue progression ;

3) Intelligent bioreactor or adjustable protocol: biophysical stimuli should be adjusted according to changes of tissue quality resulting from tissue progression over time;
4) Mechanical characterization protocol: the methods used to evaluate tissue progression over time could affect tissue progression.

To fulfill objective 2, we designed a stimulation protocol to investigate whether applying stress-relaxation tests to evaluate tissue progression over time affect this tissue progression or not. We conducted a 3-day experiment, based on this stimulation protocol, on freshly extracted tendons. The tendons were divided into two groups: the first group underwent no stress-relaxation test (0 relaxation) and the other group underwent 24 relaxation tests each day.

The results showed that applying stress-relaxation tests at physiological amplitude can modify tissue progression over time. The changes in modulus, a representative variable for mechanical properties, over the 3-day experiment were significantly different between the two groups. There was a decrease in mechanical properties of the 24-relaxation group even at the very beginning of the experiment.

Since our mechanical characterization method was in-line and non-destructive, we were able to conduct structural characterization after mechanical test. However, the difference between the structures of the two groups was not significant. The results of our experimental study approved the conclusion of the review article. In fact, we proved that mechanical characterization protocol, as an important parameter in stimulation protocol, affects live tissue progression as a result of its macro-mechanical behavior.

Résumé

Dans cette section, un résumé du travail accompli dans ce projet pour réaliser les objectifs sont présentés.

Les deux objectifs de cette étude étaient:

1) De revoir la littérature à propos de la viscoélasticité et de la viscoplasticité, ainsi que de la façon dont ces deux comportements affectent la réponse des tissus vivants aux stimulations mécanobiologiques;

2) D'investiguer si des essais diagnostiques d'amplitude physiologique affectent la réponse de tissus vivants à des stimulations mécaniques.

Un article de type "revue" a été écrit pour accomplir le premier objectif. Dans cet article, nous mentionnons que les tissus sont exposés à deux comportements macro-mécaniques : la viscoélasticité et la viscoplasticité. La viscosité des tissues vient des pertes frictionnelles reliées au contenu en eau de la matrice extracellulaire (MEC) et/ou du déplacement relatif des unités de collagènes. L'élasticité des tissus provient de l'extension réversible du collagène mais aussi du glissement relatif des unités de collagène les unes par rapport aux autres. Enfin, la plasticité est le résultat de l'extension non-réversible du collagène (associée à de grandes amplitudes de chargement ou à des chargements de grandes répétitions) menant à des micro-dommages dans la MEC ou à un glissement non-réversible du collagène. Dans les tissus vivants, la viscosité se combine avec l'élasticité ou la plasticité dépendamment de la qualité du tissu (combinaison des propriétés structurelles, compositionnelles et mécanique) et du chargement appliqué.

La viscoélasticité et la viscoplasticité affectent la réponse des tissus au chargement appliqué. Cette réponse inclus la réponse de la MEC et la réponse des cellules. Brièvement, un chargement appliqué sur la MEC est réduit de façon à être ressenti par les cellules via des mécanismes de mécanotransduction. Même si un chargement constant est appliqué macroscopiquement au tissu, le chargement local ressenti par les cellules pourrait varier dans le temps à cause de la viscoélasticité et de la viscoplasticité. La relaxation de contrainte et le fluage sont des exemples de cette situation. C'est ainsi que la réponse cellulaire (réparation ou dégradation de la MEC) varie aussi dans le temps et affecte la qualité de la MEC. En conséquence, les

comportements macro-mécaniques que sont la viscoélasticité et la viscoplasticité sont affectés.

Ainsi, nous concluons qu'il est essentiel de considérer la viscoélasticité et la viscoplasticité des tissus lors du développement d'un protocole de stimulation pour la recherche *in vitro* et les applications cliniques *in vivo*. En d'autres mots, la progression pourrait être affectée par des paramètres du protocole de stimulation à cause de ces deux comportements. Les paramètres qui doivent être mis en lumière sont :

5) Type de contrôle pour les stimulations: Les expérimentations menées sous un contrôle en contrainte affectent la progression des tissus différemment que les expérimentations menées sous un contrôle en déformation;

6) Histoire du chargement: Des périodes de repos affectent la progression des tissus;

7) Bioréacteurs intelligents ou protocoles ajustables: Les stimuli biophysiques devraient être ajustés selon les changements dans la qualité des tissus résultant de la progression des tissus dans le temps;

8) Protocole de caractérisation mécanique : Les méthodes utilisées pour évaluer la progression des tissus dans le temps pourraient affecter la progression des tissus.

Pour répondre à l'objectif 2, nous avons conçu un protocole de stimulation afin d'investiguer si l'application d'essais de relaxation de contrainte pour évaluer la progression des tissus dans le temps affecte ou non la progression des tissus. Nous avons réalisé une expérimentation de trois jours, basée sur ce protocole de stimulation, sur des tendons fraîchement extraits. Les tendons ont été divisés en deux groupes : Le premier groupe n'a subi aucun essai de relaxation de contrainte (0 relaxation) tandis que le second groupe a été soumis à 24 essais de relaxation de contrainte quotidiennement.

Les résultats ont démontré que l'application d'essais de relaxation de contrainte d'amplitude physiologique peut modifier la progression des tissus dans le temps. Les changements de module, une variable représentative pour les propriétés mécaniques, étaient significativement différents dans les deux groupes, et ce, sur les trois jours d'expérimentation. Il y avait une diminution relative dans les propriétés mécaniques du groupe de 24 relaxations dès le début de l'expérimentation.

Puisque notre méthode de caractérisation était « en ligne » et non-destructive, nous avons pu réaliser des essais de caractérisation structurale après les tests mécaniques. Toutefois, la différence entre les deux groupes était non significative.

Les résultats de notre étude expérimentale appuient les conclusions de l'article de type « revue ». En fait, nous avons démontré que le protocole de caractérisation mécanique affecte la progression des tissus vivants à cause de ses comportements macro-mécaniques.

6.2 Contributions

This section outlines the original contributions of this project.

Since tissue mechanobiology is an interdisciplinary field, it is difficult to have all the knowledge from biology and mechanics. Therefore, in the review article, we tried to link these two disciplines. We highlighted the viscoelasticity and viscoplasticity of live tissue to notify about the effect of these behaviors on live tissue response to biophysical stimulations. This was the first original contribution of this project.

Moreover, we found that using diagnostic tests, even at physiological amplitude, could affect tissue progression over time. Therefore, our answer to this paradoxical question: *"How can we measure the tissue progression over time if it responds to our measurement methods?"* is to use the stimulation itself to observe tissue progression rather than diagnostic tests. It was the second original contribution.

Finally, as the last novelty, we adapted a new method at Biometiss to characterize cell quality with fluorescent microscopy imaging of tendon cross-sections in confocal

microscope. We achieved the desired thickness of samples (50 micron) with cryostat sectioning. However, this method should still be improved to get publishable results.

Contributions

Cette section met en relief les contributions originales de ce projet.

Puisque la mécanobiologie est un domaine interdisciplinaire, il est difficile d'avoir toute la connaissance de la biologie et de la mécanique. Ainsi, dans l'article de type « revue », nous avons tenté de relier ces deux disciplines. Nous avons mis en lumière la viscoélasticité et la viscoplasticité des tissus vivants pour souligner l'effet de ces comportements sur la réponse des tissus vivants aux stimuli mécaniques. Ceci était la première contribution originale du présent projet.

De plus, nous avons démontré qu'utiliser des essais diagnostiques, même d'amplitude physiologique, peut affecter la progression des tissus dans le temps. C'est pour quoi, notre réponse à la question paradoxale : « *Comment pouvons-nous mesurer la progression d'un tissu dans le temps s'il répond à nos méthodes de mesure?* » consiste à utiliser la stimulation elle-même pour observer la progression d'un tissu plutôt que des essais diagnostiques. Ceci constitue notre deuxième contribution originale.

Finalement, comme dernière nouveauté, nous avons adapté une nouvelle méthode dans Biométiss pour caractériser la qualité des cellules à l'aide d'images de sections transversales de tendon prises sous microscopie à fluorescence. Nous avons obtenu l'épaisseur désirée des échantillons (50 microns) à l'aide d'un cryostat. Toutefois, cette méthode doit toujours être améliorée afin d'obtenir des résultats publiables.

6.3 Limitations

This section describes the limitations which restricted and decreased some potential results.

First, the bioreactor occasionally did not provide the exact displacement it was assigned to. Moreover, in some cases the load cell and/or encoder were malfunctioning. In all cases the related tendons were discarded.

Second, there were some challenges for structural characterization of tendon:

- For quantitative structural characterization using NI-Vision software, there was a difficulty to set an appropriate and repetitive contrast, and consequently proper fitting of black-red image with the origin image. This would make a significant error in quantifying tissue structural quality.
- For semi-quantitatively structural characterization using the Bonar-Movin scoring scale, there were some difficulties for scoring the images. The scoring scale was based on the most damaged tissues while most of the samples from both groups (0-relaxation and 24-relaxation tests) were not much damaged. Moreover, because of some imperfections in tissue preparation for OM microscopy, there were some damage signs (e.g. collagen partial tears, or large spaces between fibers) which were not the result of stimulation. These difficulties led to non-repetitive scorings and/or mistaken scorings.

Finally, as the framework of this thesis, we conducted the experiment based on only one protocol and on only one tissue quality i.e. freshly extracted tendons. To have a better understanding of how diagnostic tests affect tissue progression other protocols could be designed, e.g. using more or less repetitions of stress- relaxation tests each day. Moreover, the protocols could be tested on damaged tissue to investigate whether the effect of diagnostic test on tissue progression changes or not.

Limites

Cette section décrit les limites qui ont affecté négativement des résultats potentiels.

Premièrement, il arrivait que le bioréacteur ne fournisse pas le déplacement exact demandé. De plus, il arrivait que la cellule de force et/ou l'encodeur fonctionne mal. Dans tous ces cas, les tendons affectés étaient exclus de l'analyse.

Deuxièmement, nous avons rencontré des défis dans la caractérisation structurale des tendons:

- Pour la caractérisation structurale quantitative à l'aide du logiciel NI-Vision, il était difficile de fixer un contraste approprié et répétitif et, conséquemment, de transformer adéquatement l'image originale en image rouge et noire. Ceci créait des erreurs significatives lors de la quantification de la qualité structurale des tissus.

- Pour la caractérisation structurale semi-quantitative à l'aide de l'échelle de Bonar-Movin, nous avons rencontré certaines difficultés à noter les images. L'échelle de notation était basée sur les tissus les plus endommagés alors que la majorité des échantillons des deux groupes (0 et 24 relaxations) n'était pas si endommagée. De plus, à cause de certaines imperfections dans la préparation des tissus pour la microscopie optique, il y avait des signes de dommages (e.g. déchirures partielles du collagène ou larges espaces entre les fibres) qui n'étaient pas le résultat de la stimulation. Ces difficultés ont mené à des notes non répétitives ou erronées.

Finalement, dans ce projet, nous avons mené une expérimentation basée sur seulement un protocole et une qualité de tissu soit des tendons fraîchement extraits. Pour obtenir une meilleure compréhension de comment les essais diagnostiques affectent la progression des tissus, d'autres protocoles pourraient être conçus, par exemple en utilisant plus ou moins de répétitions des essais de relaxation de contrainte chaque jour. De plus, les protocoles pourraient être testés sur des tissu endommagés pour investiguer si l'effet des essais diagnostiques sur la progression de tissus changerait ou non.

6.4 Future work

There are several potential studies which could be pursued following this research study in order to extend our understanding of tissue mechanobiology.

First, as mentioned in the previous section, we conducted the stimulation protocols including 0 or 24 relaxation tests. We could design other protocols by changing the parameters of the stimulation protocol e.g. changing the numbers and/or duration of relaxation test, shortening or lengthening the rest periods, etc. Moreover, we could conduct the experiment on damaged tissue instead of healthy one. By applying these new experimental conditions, whether the effect of diagnostic tests on tissue progression increases or decreases or remains constant could be investigated.

Second, by using the results of this study, the stimulation protocols could be optimized. For example, since diagnostic tests could modify tissue progression over time, they should be considered as part of the stimulation not to introduce more energy to tissues. As another example, considering that stress-controlled and strain-controlled experiments modify tissue progression in different ways, we could design stimulation protocols with a combination of both control types. Using each control type at different time points makes it possible to change the type of tissue progression in order to achieve the desired tissue quality. These optimized stimulation protocols could be used to improve tissue quality or to optimize the healing of damaged tissues.

Finally, in order to be able to conduct *in vitro* experiments in *in vivo* conditions, *in vitro* stimulation parameters should be translated into *in vivo* parameters. This arises many questions and thus needs to be deeply studied. Some parameters, such as frequency and rest periods, could merely be applied to *in vivo* i.e. the frequency of *in vitro* stimuli and the rest periods between *in vitro* stimuli could simply be adjusted to daily or occupational activities. For some other parameters, such as stimulation control type (stress-control *vs* strain-control), the corresponding situation in daily or occupational activities (such as walking, and repetitive manual handling) is not fully clarified. Therefore, it is challenging to translate *in vitro* studies to *in vivo* applications. Consequently, more investigation is required. For example, we could investigate

whether: passive motions could be considered as a strain-controlled *in vivo* experiment, active motions could be considered as stress-controlled *in vivo* experiment, etc.

The results of this work could be helpful in developing methods of rehabilitation and improving live tissues quality through the design of more optimized treatment strategies based on mechanobiology for both bioreactor experiment and clinical application.

Travaux futurs

Il y a plusieurs études potentielles qui pourraient être réalisés pour poursuivre cette étude afin d'approfondir notre compréhension de la mécanobiologie tissulaire.

Premièrement, tel que mentionné dans la section précédente, nous avons réalisé des protocoles de stimulation incluant 0 et 24 essais de relaxation de contrainte. Nous pourrions concevoir d'autres protocoles en changeant les paramètres de stimulation, par exemple en changeant le nombre et/ou la durée des essais de relaxation de contrainte, en raccourcissant ou en allongeant les périodes de repos, etc. De plus, nous pourrions réaliser l'expérimentation sur des tendons endommagés plutôt que sur des tendons sains. En appliquant ces nouvelles conditions expérimentales, il serait possible d'investiguer si l'effet des essais diagnostiques sur la progression des tissus diminue, augmente ou demeure identique.

Deuxièmement, en utilisant les résultats de cette étude, les protocoles de stimulation pourraient être optimisés. Par exemple, puisque des essais diagnostiques pourraient influencer la progression des tissus dans le temps, ils devraient être considérés comme faisant partie du protocole de stimulation parce qu'ils induisent de la nouvelle énergie dans les tissus. Comme autre exemple, considérant que des expérimentations sous contrôle en contrainte *vs* en déformation influences différemment la progression des tissus, nous pourrions concevoir des protocoles combinant les deux types de contrôle. En utilisant chaque type de contrôle à différents moments rendrait possible de changer la progression du tissu afin d'obtenir la qualité tissulaire désirés. Ces protocoles de

stimulation optimisés pourraient être utilisés pour améliorer la qualité des tissus ou pour optimiser la guérison des tissus endommagés.

Finalement, afin de pouvoir réaliser des expérimentations *in vitro* sous des conditions *in vivo*, les paramètres de stimulation *in vitro* doivent être traduits en paramètres de stimulation *in vivo*. Ceci soulève plusieurs questions et nécessite donc d'être étudié en profondeur. Quelques paramètres pourraient simplement être appliqués *in vitro*. Par exemple, la fréquence de stimuli *in vitro* et les périodes de repos entre les stimuli *in vitro* pourraient simplement être ajustées aux activités quotidiennes ou au travail. Pour d'autres paramètre comme le type de contrôle (contrôle en contrainte vs contrôle en déformation), les situations correspondantes dans les activités quotidienne ou au travail (e.g. marcher, réaliser une tâche répétitive au travail) ne sont pas totalement clarifiées. Donc, il est difficile de traduire les études *in vitro* en applications *in vivo*. En conséquence, plus d'investigation sont requises. Par exemple, nous pourrions investiguer si des mouvements passifs peuvent être considérer comme des expérimentations sous contrôle de déformation *in vivo*, si des mouvements actifs peuvent être considérés comme des expérimentations sous contrôle en contrainte *in vivo*, etc.

Les résultats de ce travail pourrait être bénéfiques au développement de méthodes de réadaptation et à l'amélioration de la qualité des tissus via la conception de stratégies optimisées basée sur la mécanobiologie tant pour des expérimentations en bioréacteurs que pour des applications cliniques.

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